Final Report On The Freeze-Drying Of Cellular Materials: Cryopharm Research Report

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Final Report On Freeze Drying of Cellular Components

The purpose of this summary is to provide an overview of the principles learned during research under Cryopharm's red cell lyophilization program. In addition, this summary will provide an assessment of the issues related to remaining technical hurdles to be addressed for cellular freeze-drying.

Review of Research Program

A copy of a recent publication in *Advances in Low-Temperature Biology* (vol. 2) is included in the Appendix section. This review article summarizes Cryopharm's research efforts on the freeze-drying of red blood cells. The research described covers the period from June of 1987 to March of 1992 and includes a detailed discussion of those principles learned during this period.

Remaining Technical Hurdles In Cellular Freeze-Drying

1. Stability as a function of moisture level and storage temperature.

2. Selection of an appropriate lyophilization container: Maintenance of sterility in non-sterile environments.

3. Conditions and media for rehydration.

Stability As A Function Of Moisture Level And Storage Temperature

Utilizing the principles of water replacement and glass transition theories, it has been possible to define conditions under which 70-80% of the water present in a sample may be removed while still maintaining normal cellular properties. The water remaining in these samples is not available in a form which is capable of acting as a solvent or catalyst for biological or chemical processes as long as the temperature is maintained below the glass transition temperature (T_G'). The stability of a freeze-dried preparation is thus defined by the residual moisture content and the storage temperature. Drying samples further raises the value of T_G'. Excessive drying runs the risk, however, of overwhelming the capacity of the water replacement agents to functionally replace cell associated water. The result is unacceptable levels of cell damage.

In order to develop preparations with extended shelf-lives at room temperature or higher, it will be necessary to establish a series of stability, temperature, and residual moisture surfaces. Such surfaces will be determined by the buffer constituents. By comparing preparations devised by application of the buffer formulation procedures described in the Appendix section, it should prove possible to define a set of conditions under which acceptable cell quality can be maintained within the desired temperature ranges. Such studies will be crucial in establishing the feasibility of storage for prolonged periods at elevated temperatures. Such studies will also provide a key understanding of the playoff between cell quality and moisture level.

Selection Of Lyophilization Container

The preparation of lyophilized cellular samples which maintain sterility of the sample requires handling in a sterile environment. This requirement arises because the sample must be permitted an open orifice for diffusion of water out of the sample. Constrictions on this orifice to provide sterility can also restrict the flow of water vapor and prohibit drying. In order to develop a method for carrying out sample drying in a non-sterile environment, a suitable material for closure of the drying orifice must be developed. Such a material would permit free movement of gases and water vapor but be impermeable to liquid water and microbiological contaminants. Such material would also have to be compatible with the cellular material that will come in contact with the surface. A container constructed of this material will be necessary to develop lyophilization cycles on reasonable time scales using equipment and preparative methods carried out in non-sterile environments.

Conditions And Media For Rehydration

Successful rehydration and appropriate post-rehydration sample handling conditions will be determined by the selected lyophilization buffer constituents and the ability of the cells to withstand rehydrateion induced osmotic stresses (see Appendix section). Osmotic stresses incurred during rehydration may lead to cell lysis, inducing a need to wash reconstituted samples in order to remove cell lysate. In addition, the need to avoid infusion of large volumes of lyophilization buffer constituents may also make a washing procedure necessary. These issues may be best assessed by evaluating the behavior of reconstituted versus reconstituted and washed cells *in vivo* using an appropriate animal model. Such a model system will also permit the evaluation of alternatives to manual washing such as filtration to remove buffer constituents prior to infusion. Once again, appropriate conditions will need to be defined according to the desired end use applications and requirements.

General Conclusions

The realization of lyophilized cell preparations for use in transfusion medicine applications appears to be within reach. The limitations of such systems and their utility to real appplications will be dictated by the resolution of several practical issues regarding lyophilization containers, rehydration methods, and residual moisture sensitivity relative to storage temperature and duration of storage.

Direct application of those methods developed by Cryopharm during the course of its Navy sponsored research program in this area should resolve these issues and establish the likelihood of achieving lyophilized cellular products for military and civilian use.

APPENDIX

FREEZE-DRYING OF RED BLOOD CELLS

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OUTLINE

1. Introduction 54
2. Metabolic Function 63
3. Hemoglobin Preservation 67
4. Cytoskeletal Studies 72
5. Membrane Studies 79
6. General Hypotheses 81
7. Current Status of Development 85

References 96

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1. INTRODUCTION

When first considered, the concept of freeze drying of living cells in a manner that maintains viability of the cells evokes thoughts of magic rather than science. Yet, the science of freeze drying of living cells has been described in the literature for over 40 years (Benedict et al., 1958; Fry and Greaves, 1951; Naylor and Smith, 1946; Proom and Hemmons, 1949). Attempts at lyophilizing mammalian cells date as far back as 1949 (Polge et al., 1949; Sherman, 1954). Meryman and Kafig (1959) described experiments involving the lyophilization of spermatazoa. Larson and Graham (1976) also described procedures for lyophilizing spermatazoa. More recently, Greiff and Milson (1980) described in great detail experiments involving the lyophilization of human lymphocytes. Each of these cases, however, is more notable because of their limitations and shortcomings than for their successes in applying to mammalian cells the priniciples of freeze drying that have worked for proteins and prokaryotic cells.

Meryman's intial report (Meryman and Kafig, 1959) of success in freeze drying of mammalian cells was followed by years of recantation of the reported success. Larson's experiments with spermatazoa (Larson and Graham, 1976) indicated that drying could be successfully accomplished as long as residual moisture contents did not fall too low (below 7–12%). Even under these circumstances, however, successful insemination of cattle was reported in limited cases. Despite this initial success, very little was ever reported as follow-up to this work. Greiff's report on the lyophilization of human lymphocytes (Greiff and Milson, 1980) carefully avoids the issue of viability by stating that only certain functions of the cells were preserved following drying and subsequent rehydration.

If one is to accept the fact that lyophilization of non-mammalian cells has been successfully accomplished, then the apparent lack of success in lyophilizing mammalian cells must be due to either the inherent differences in the cells themselves or due to the inadequate understanding of the mechanisms of preservation of any cell under conditions of dehydration. It was this latter possibility that drove us in the summer of 1987 to attempt to lyophilize human erythrocytes.

Our first attempts at lyophilization focused on the use of carbohydrates exclusively. The use of carbohydrates for preservation of proteins, bacteria, etc. subjected to freeze drying has been broadly discussed in the literature (Scott, 1958, 1960; Heckly, 1961). The proposed role that the carbohydrates play in these systems has varied

from worker to worker. One popular concept portrays the carbohydrate as a water replacement molecule (Clegg et al., 1982; Beardmore, 1979). According to this view, carbohydrates manifest their action in dry systems by replacing water hydrogen bonds through hydrogen bonding of carbohydrate hydroxyl groups to membranes and proteins subjected to dehydration. This view is perhaps most clearly described in the work of John and Lois Crowe (Crowe and Crowe, 1984; Mouradian et al., 1984; Crowe et al., 1984). Through studies using model membrane systems comprised of phospholipid vesicles and reconstituted sarcoplasmic reticulum, Crowe et al. were able to clearly demonstrate the potential and consequences of hydrogen bonding between carbohydrates and membranes subjected to desiccation. Later, this work was extended to lyophilized protein systems by Carpenter and Crowe with comparable results (Carpenter et al., 1984, 1987a,b).

In our initial experiments, samples of washed red blood cells were mixed in solutions containing varying concentrations of carbohydrates. The suspensions were mixed to provide a hematocrit at lyophilization of 10–15%. These suspensions were transferred to flasks that were frozen in liquid nitrogen (−196°C). All samples were lyophilized on a Labconco model 4.5 lyophilizer operating at 5 to 10 mtorr vacuum and a condenser temperature of −56°C. Samples were allowed to dry for up to 24 h until they were crystalline in appearance and brittle to touch. Residual moisture contents of samples dried in this fashion varied between 5–10% as measured by the Karl–Fischer method. All samples were rehydrated at 37°C with a solution containing 700 mM sucrose in phosphate-buffered saline. Rehydrated samples were examined via microscopy to determine the level of distinct and intact cells with intact cell membranes. This was reported as % cell recovery.

As Figure 1 indicates, in the absence of any carbohydrate, only massively fused and aggregated cells could be observed under the microscope. Intact and individual cells were absent from these samples. In addition, the appearance of the rehydrated samples was very dark brown, indicative of severe oxidation of the hemoglobin. Spectrophotometric evaluation of the samples confirmed this (Table 1). Samples containing carbohydrate were distinctly different. In these cases, intact and individual cells could be observed at levels that varied according to the type of carbohydrate that was used and its concentration. The amount of protection varied, but generally reached a maximum when the starting concentration of the carbohydrate was

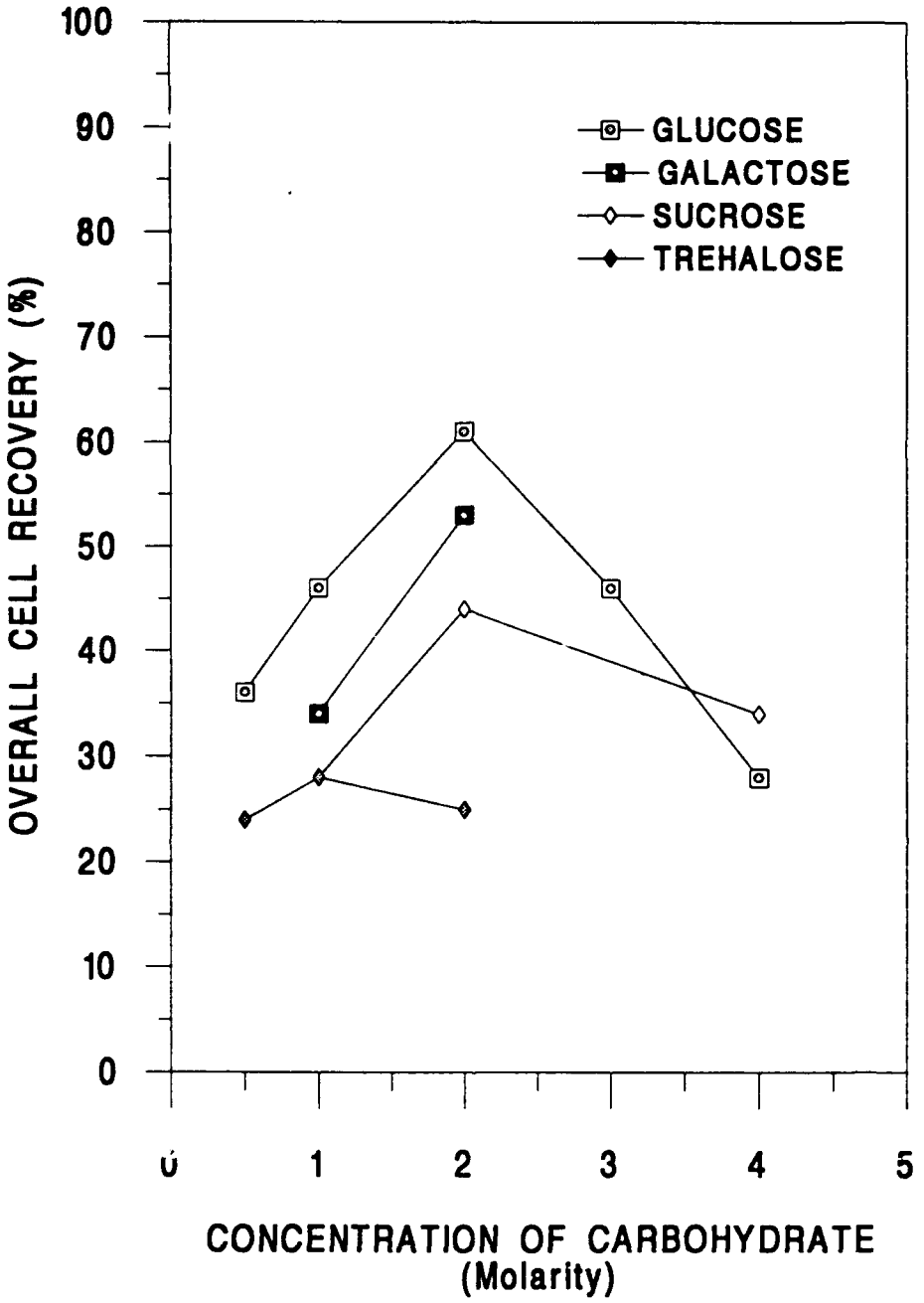


Figure 1. Percent cell recovery as a function of carbohydrate concentration. All the different carbohydrates evaluated demonstrated concentration-dependent lyoprotective effects on the cells. The maximum cell recovery was observed at 2 M concentration independent of the carbohydrate.

2 M and actually decreased at higher concentrations. The carbohydrates appeared to have functioned in a manner that prevented cell fusion, which was observed in samples without the carbohydrate. This behavior was consistent with the descriptions of carbohydrate action in inhibiting or preventing fusion of liposomes (Crowe and Crowe,

Table 1. Protection of red blood cell hemoglobin from oxidative damage by different carbohydrates. In the absence of carbohydrate, a significant amount of methemoglobin was converted to denatured hemoglobin, hemichrome that is incapable of oxygen transport.

Carbohydrates (2 M)	Oxyhemoglobin (%)	Methemoglobin (%)	Hemichrome (%)
None	0	90	10
Ribose	93.1	5.4	1.5
Mannose	94.2	6.0	0
Fructose	98.0	1.3	0.7
Sorbose	56.9	40.9	2.3
Galactose	81.0	17.3	1.7
Xylose	96.7	3.6	0
Glucose	98.1	1.8	0.1

1984). Unfortunately this protection did not extend to retention of cellular contents as the cells appeared to have leaked nearly all of their hemoglobin. Unlike the control samples, however, this hemoglobin was preserved in its native oxyhemoglobin form (Table 1). This preservative capacity of carbohydrates for dried hemoglobin suspensions had previously been observed by Labrude et al. when dealing with isolated, non-cellular hemoglobin preparations (Labrude et al., 1980; Labrude and Vigneron, 1982).

The data in Figure 1 indicated that, in our initial experiments and contrary to what we had anticipated, monosaccharides were generally more effective than disaccharides in providing recovery of cells with intact membranes. This result appeared to us at first to be contradictory to literature reports that indicated greater lyoprotective properties for disaccharides. The answer to this question was already in the literature. In 1986, Womersley et al. reported that, in order for membrane preservation to be effective, the carbohydrate had to be present on both sides of the membrane (Womersley et al., 1986). Failure to achieve this would greatly reduce the capacity of the carbohydrate to protect the membrane against desiccation-induced damage. This feat is relatively easy to accomplish with phospholipid vesicles, which can be made *de novo*, but it is considerably more difficult to accomplish when dealing with a pre-formed and intact cell. Disaccharides, under these conditions, would not be expected to penetrate the cells. Monosaccharides, to the contrary, are capable of entering the cell via the glucose transporter. It was no surprise then, given this consideration, that our results showed glucose to be the

Table 2. Influence of polymers on hemoglobin recovery after lyophilization.

Polymer	Hemoglobin Recovery (%)	MCHC[a] (g/dL)
PVP 10K	30.1 ± 4.1	20.9 ± 3.1
PVP 24K	52.7 ± 6.3	27.4 ± 4.3
PVP 40K	61.4 ± 4.1	25.7 ± 9.2
Dextran 10K	26.5	ND[b]
Dextran 40K	25.9	ND
Dextran 80K	20.2	ND

[a]MCHC, mean cell hemoglobin concentration.
[b]ND, not determined.
In each of the above studies the concentration of polymer and carbohydrates were kept at 12.8% and 2.0M respectively.

most effective agent while the disaccharides were relatively less effective (trehalose, sucrose) or completely ineffective (maltose).

A second point evident from these initial studies was that the level of recovery, although considerably better than when no protectant was used, was not even close to yielding cells that were comparable to the starting cells. Cells recovered following this process resembled ghosts and although they were distinct as individual cells with minimal hemoglobin inside, it was very evident that severe alterations had occured. These cells were inherently unstable and quickly surrendered their remaining hemoglobin upon standing or when they were washed with isotonic saline solutions. We were pleased to find, however, that reconstitution and washing of the cells in hypertonic media afforded greater stability. This stability could be further enhanced when polymers such as dextran, hydroxyethyl starch, or polyvinyl-pyrrolidone were included in the resuspending media. Based on these results, we decided to take a step back and attempt to lyophilize samples in the presence of solutions containing both carbohydrates and polymers. The results were astounding (Table 2). Samples showed

Figure 2. Microphotographs of lyophilized human red blood cells at various stages of washing. (A) Lyophilized cells in a dry matrix; (B) Cells at reconstitution appear spherocytic, but maintain internal hemoglobin content; (C) After one wash in isotonic media containing 10% PVP, the cells return to more normal morphology; (D) After an additional wash in the same medium, the majority of cells appear as discocytes. Recovery at this stage was generally 40–50% of starting sample. Samples were photographed under Nomarski optics at 400×.

A
B
C
D

improved levels of recovery of both intact cells and cells with hemoglobin associated with them (reported as % hemoglobin recovery). When these cells were washed with media containing polymer, not only did the cell recovery and hemoglobin recovery increase dramatically, but the cell appearance also became increasingly better, even approaching normal discocytic morphology (Figure 2). Even under these conditions, however, the cells still exhibited a reduced stability and lysed completely upon washing in isotonic saline solutions. It was at this point that we rediscovered a report by Meryman dating from 1960 that described his own attempts to lyophilize red blood cells (Meryman, 1960).

In that early report, Meryman described the successful freeze drying of human and rat red cells using polyvinylpyrrolidone (PVP) as a protectant. The original paper had indicated that the dried rat red cells had even demonstrated acceptable cell survival *in vivo*! It was amazing to us that there had been no follow-up on this report for nearly 30 years. Our curiousity was quenched, however, when we subsequently read the report on spermatazoa drying by Larson and Graham (1976). In a discussion section following the paper, Meryman related the story of the drying of red cells and suggested that his previous results should be reconsidered. Meryman indicated that what was actually being observed was merely a hemoglobin droplet and not an intact cell. He argued that the PVP used in the media reduced the surface tension of the solution, allowing the hemoglobin to form globules much like oil in water droplets. This behavior of polymeric compounds inhibiting hemolysis was described in greater detail many years later by Williams et al. (1983).

In the same article (Larson and Graham, 1976), however, this claim of complete membrane destruction was refuted by A. P. MacKenzie who claimed that he had successfully dried human cells under similar conditions and obtained intact, albeit unstable, cells at the end. Our own results were more consistent with those described by MacKenzie, although his method for producing these cells was never published in the literature. Meryman's original article had reported on both human and rat red cells. Our experience up to this point had been limited to human cells. We would soon discover that what was true for human cells did not hold for animal cells.

Table 3 contains a summary of results obtained using samples of animal cells and the same buffers and conditions derived for human samples. Particularly astounding were the results obtained with rat and guinea pig cells. These samples showed what at first appeared to

Table 3. Effects of freeze-drying on membrane integrity of red blood cells from different animal species. Cells were lyophilized and reconstituted according to standard procedures that had been established for human red blood cells.

Species	Results
Rat	Formed hemoglobin emulsions upon rehydration. No intact cells were present at reconstitution.
Guinea Pig	Same results as rat were obtained.
Dog	Less than 10% of intact red blood cells were obtained at the end of reconstitution. Needle-like hemoglobin aggregates were obtained. Extensive oxidative damage was seen with red blood cells. Cell hemoglobin was converted to 100% methemoglobin.

be amazingly high levels of recovery of intact cells. Samples could be washed in polymer solutions with high levels of hemoglobin retention. Under microscopic examination of the cell pellets however, there was no indication of a cell being present. Instead, only hemoglobin droplets or needle-like structures of protein were observed (Figure 3). Indeed, consistent with Meryman's descriptions, there did not appear to be any membrane associated with these hemoglobin droplets.

The results with these animal cells were distinctly different from those observed with the human cells. The reason for this difference was never fully explored, but may have arisen from differences in protein or lipid composition. Human red cell samples lyophilized in monosaccharide plus polymer and washed in an isotonic medium containing polymer exhibited high levels (40–50%) of recovery of intact cells with normal discocytic morphology. Yet, when these cells were washed in an isotonic medium without polymer, they would swell to spherocytes and lyse. There had to be some distinct reason for this behavior, and it was not that the membranes were completely destroyed.

Cellular lysis and loss of cell stability can be explained by many factors. Although the red cell is a relatively simple cell, it still requires adequate preservation of membrane lipid structure, hemoglobin structure, cytoskeletal structure, and transporter function in order to work properly. Disruption of one or more of these systems could produce the effects that we had observed. An investigation of each of these systems in the lyophilized cells was called for. This was the task that we decided to undertake in the spring of 1988.

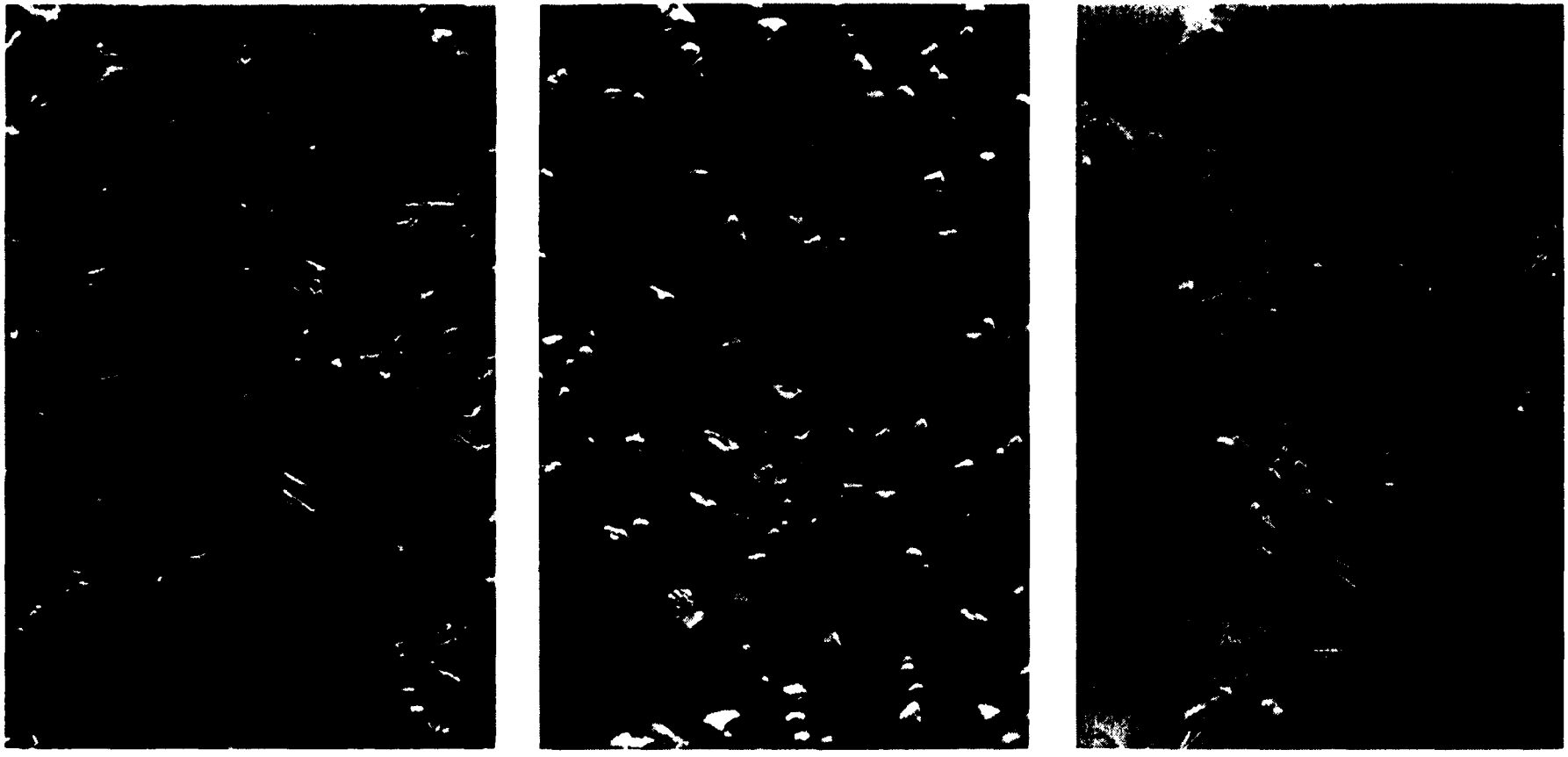


Figure 3. Microphotographs of lyophilized animal red blood cells. (A) Rat red blood cells. Although red blood cell pellets were obtained after centrifugation, indicative of high recovery of intact cells under normal circumstances, no intact red blood cells could be observed after centrifugation of the lyophilized cells. (B) Guinea pig red blood cells. (C) Dog red blood cells. Each animal cell showed distinct characteristics following lyophilization and reconstitution. Of all the different species tested, only primate and human red blood cells showed hemoglobin recovery with intact red blood cells. Cell morphology was examined using Nomarski optics at 400×.

2. METABOLIC FUNCTION

Our initial studies focused on the metabolic functioning of the cells. A breakdown in the glycolytic pathway by loss of even a single enzyme would cause glycolysis to cease and ATP production to halt. Cells

Table 4. Summary of the activities of the glycolytic enzymes in hemolysates from rehydrated lyophilized and non-lyophilized RBC.

	Enzyme activity (μmol/min/g Hb)				
Enzyme	Lyophilized	Non-lyophilized	Blood-bank sample	Normal range	Statistical significance (p)
HX	1.26 ± 0.22	1.65 ± 0.10	1.20 ± 0.12	0.98 –1.3	NS
PGI	44.7 ± 4.57	44.3 ± 2.66	48.3 ± 6.03	43.7 – 65.8	NS
PFK	12.1 ± 1.61	11.7 ± 0.97	9.73 ± 2.18	8.44 – 12.02	NS
Ald	3.59 ± 0.41	3.72 ± 0.54	2.39 ± 0.34	1.97 – 3.59	NS
TPI	1750 ± 460	2140 ± 490	2900 ± 777	2130 – 3340	$p<0.005$
G3PD	318 ± 68.4	311 ± 43.0	244 ± 72.0	238 – 346	NS
DPGM	5.34 ± 0.72	4.64 ± 0.91	8.43 ± 2.23	3.93 – 5.90	$p<0.015$
PGK	340 ± 147	340 ± 115	349 ± 47.7	212 – 341	NS
PGM	35.2 ± 5.09	38.1 ± 5.99	17.3 ± 6.70	13.9 – 38.0	NS
Eno	4.99 ± 0.99	7.60 ± 0.87	4.96 ± 0.89	4.2 – 6.58	$p<0.001$
PK	18.9 ± 5.71	21.1 ± 5.40	15.0 ± 2.14	12.5 – 17.2	$p<0.032$
LDH	231 + 29.0	190 ± 19.2	141 ± 56.4	145 – 203	$p<0.001$
G6PD[a]	124 ± 1.55	14.7 ± 1.82	ND	9.90 – 13.2	NS
6PGD[a]	11.1 ± 0.99	10.0 ± 1.09	ND	7.27 – 10.0	NS
TA[a]	0.97 ± 0.21	1.10 ± 0.34	ND	0.78 – 1.32	NS
TK[a]	0.68 ± 0.13	0.93 ± 0.66	ND	0.50 – 1.03	NS

[a] Enzymes of the Pentose Phosphate Shunt.

ND, not detected p, probability for comparisons between lycophilized and non-lyophilized. Data represent the mean ± sd for six samples. Data from blood bank stored RBC are included for comparison with lyophilized-rehydrated RBC. Blood bank samples (total number 3) were stored at 4°C for at least 10 days before being analysed. Abbreviations: lyo, lyophilized; N-lyo, non-lyophilized; BB, Blood bank; N-R, normal range; ND not detected; NS, not significant (comparisons were made between lyophilized and non-lyophilized RBC); HX, hexokinase (ATP:D-hexose-6-phosphotransferase, EC 2.7.1.1); PGI, glucose-6-phosphate isomerase (D-glucose-6-phosphate ketol-isomerase, EC 5.3.1.9); PFK, 6-phosphofructokinase (ATP:D-fructose-6-phosphate 1-phosphotransferase, EC 2.7.1.11); Ald, fructose-bisphosphate (D-fructose-1,6-bisphosphate D-glyceraldehyde-3-phosphate-lyase, EC 4.1.2.13); TPI, triosephosphate isomerase (D-glyceraldehyde-3-phosphate ketol-isomerase, EC 5.3.1.1); G3PD, glyceraldehyde-3-phosphate dehydrogenase (D-glyceraldehyde-3-phosphate:NAD^+ oxidoreductase (phosphorylating), EC 1.2.1.12); DPGM, bisphosphoglycerate mutase (3-phospho-D-glycerate 1,2-phosphomutase, EC 5. 4.2.4); PGK, phosphoglycerate kinase (ATP:3-phospho-D-glycerate 1-phosphotransferase, EC 2.7.2.3); PGM, phosphoglycerate mutase (D-phosphoglycerate 2,3-phosphomutase, EC 5.4.2.1); Eno, enolase (2-phospho-D-glyceratehydro-lyase, EC 4.2.1.11); PK, pyruvate kinase (ATP:pyruvate O^2-phosphotransferase; EC 2.7.1.40) LDH, D-lactate dehydrogenase (lactate:NAD^+ oxidoreductase, EC 1.1.1.28); G6PD, glucose-6-phosphate dehydrogenase (D-glucose-6-phosphate:$NADP^+$1-oxidoreductase, EC 1.1.1.49); 6PGD, phosphogluconate dehydrogenase (6-phospho-D-gluconate:NAD(P)$^+$2-oxidoreductase, EC 1.1.1.43); TA, transaldolase (sedoheptulose-7-phosphate:D-glyceraldehyde-3-phosphate glycerone transferase, EC 2.2.1.2), TK, transketolase (sedoheptulose-7-phosphate:D-glyceraldehyde-3-phosphate glycoaldehyde transferase, EC 2.2.1.1).

depleted of ATP essentially shut down all ATP-dependent functions, an event that leads to swelling of the cells and increased osmotic fragility. In collaboration with Dr. Kouchi Tanaka and Dr. Charles Zerez at Harbor-UCLA hospital, we examined the metabolic integrity of lyophilized cells. This study was published in February of 1992 (Goodrich et al., 1992).

Table 4 shows data on enzyme activities measured for the hemolysates of lyophilized and non-lyophilized samples. Comparison is provided to comparable values obtained for freshly drawn blood and samples stored in a blood bank. The results of this work clearly showed that the activities of most of the enzymes in question remained within ranges comparable to untreated samples. For triosephosphate isomerase and pyruvate kinase, there was a distinct reduction in the activity of the enzymes. Even in these cases, however, there did not appear to be a significant difference between the enzyme activities of the lyophilized cells and those that had been stored at 4°C in a conventional blood-bank refrigerator. With pyruvate kinase, the activity of the lyophilized samples was greater than that of the sample stored in the blood-bank.

Table 5 Comparison of the levels of glycolytic intermediates in lyophilized-rehydrated and fresh, non-lyophilized RBC.

	Concentrations of intermediates (nmol/g Hb)			
Intermediates	Lyophilized	Non-lyophilized	Normal values	Statistical significance (P[a])
G6P	49.8 ± 72.1	76.5 ± 102	100 ± 28.0	NS
F6P	0.92 ± 2.26	3.05 ± 7.47	15.6 ± 6.30	NS
FDP	760 ± 425	149 ± 179	4.70 ± 1.60	NS
DHAP	1770 ± 687	174 ± 147	37.5 ± 3.10	p<0.012
GAP	112 ± 46.8	44.9 ± 43.5	9.38 ± 6.30	NS
2,3-DPG	3152 ± 938	9633 ± 2640	13500 ± 2000	p<0.004
3PG	611 ± 210	134 ± 56.1	122 ± 28.0	p<0.006
2PG	338 ± 252	216 ± 165	31.3 ± #13.0	p<0.046
PEP	216 ± 104	67.5 ± 50.8	50.0 ± 16.0	p<0.01
Pyr	170 ± 52.5	193 ± 125	84.4 ± 25.0	NS
Lact	6032 ± 2730	9495 ± 3542	1140 ± 370	NS
ATP	1758 ± 392	3875 ± 780	3220 ± 280	p<0.008
ADP	1743 + 316	700 + 133	409 + 56.0	p<0.003
AMP	2370 ± 343	204 ± 125	134 ± 25.0	p<0.001

Data represent the mean ± SD for six samples. Normal values are included in the table for comparison with present data.

[a] probability for comparisons between lyophilized and non-lyophilized RBC.

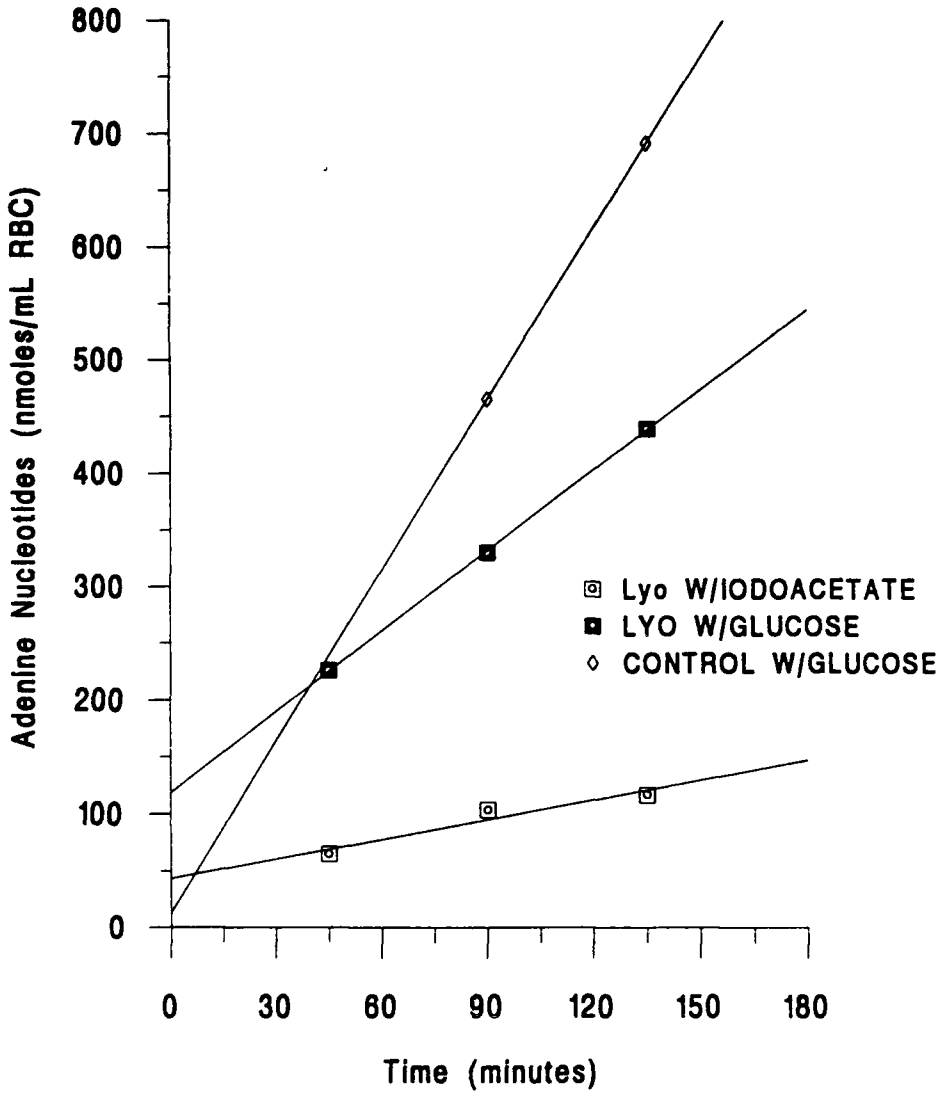


Figure 4. The rate of adenine nucleotide synthesis was measured by following the incorporation of ^{14}C-labeled adenine into the adenine nucleotide pool. Because all red blood cell samples had endogenous glucose and exhibited low rates of ^{14}C-adenine incorporation in the absence of exogenous glucose, a control was added in which incorporation was measured in the presence of 1.0 mM iodoacetate, an inhibitor of glycolysis. This allowed the assessment of 'background' ^{14}C-adenine incorporation (i.e. under conditions of total inhibition of glycolysis) by subtracting ^{14}C-adenine incorporation in the presence of iodoacetate from incorporation in its absence. Thus, reported rates of ^{14}C-adenine were corrected for this background incorporation. Although lyophilized red blood cells had a lower rate of ^{14}C-adenine nucleotide incorporation, the ability of these cells to incorporate adenine into nucleotides is noteworthy because only freshly obtained red blood cells are capable of this function.

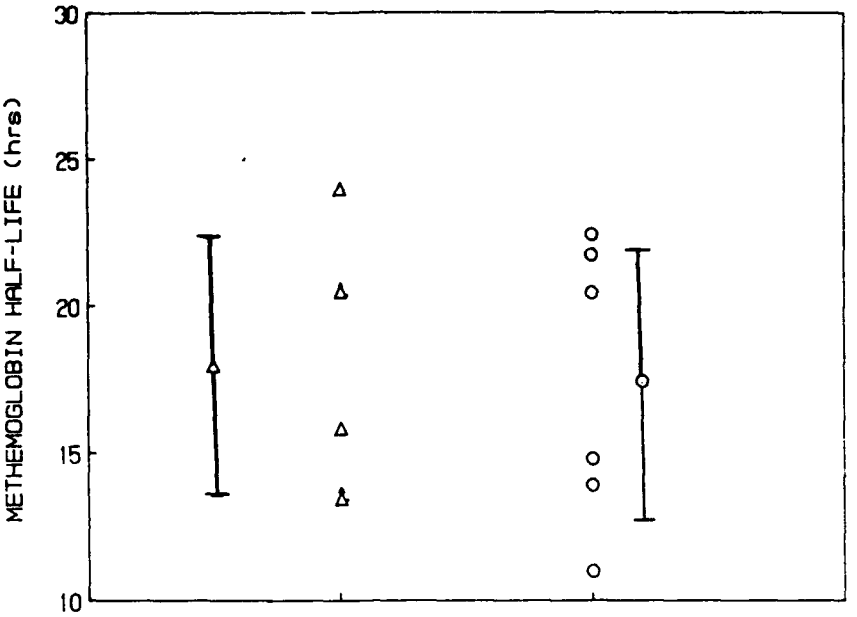


Figure 5. Rate of methemoglobin reduction (expressed as half-life) in intact lyophilized (△) and non-lyophilized red blood cells (○). The mean ±SD methemoglobin half-life for both cell types are also shown. The mean rate of methemoglobin reduction in lyophilized, red blood cells was 17.9±4.3 h, and was not significantly different from control, non-lyophilized cells, 17.4±4.8 h, p.0.05.

Measurement of the levels of glycolytic intermediates produced comparable results (Table 5). Levels of ATP and 2,3-DPG were lower in the lyophilized samples than in the control samples, but sufficient amounts of these intermediates appeared to remain to provide for viability and continuation of the ATP- and 2,3-DPG-dependent functions in the cell. Rates of metabolic functioning were directly established by examining the rate of lactate formation, the end product of glycolysis, and the rate of incorporation of radiolabelled adenine into ATP. The rate of lactate production was reduced from 10.6±2.86 μmol/g (Hb)/h in control samples to 6.60±3.58 μmol/g (Hb)/h for lyophilized samples. The rate of ATP production was reduced from 0.779±0.305 μmol/g (Hb)/h for control samples to 0.368±0.173 μmol/g (Hb)/h for lyophilized samples. The validity of the incorporation rates of ATP were checked using samples treated with iodoacetate, which poisons the process. As Figure 4 indicates, the addition of iodoacetate to the lyophilized samples shut down ATP production. The rate of methemoglobin reduction was also determined to establish the rate at which other metabolically driven processes were occuring. As Figure 5 indicates, the half-lives of methemoglobin

in control and lyophilized samples were comparable (17.4±4.8 versus 17.9±4.3 h, respectively) indicating that this metabolically dependent process was not compromised.

None of the changes observed in the metabolic functioning of the lyophilized cells were indicative of a pathological state of the cells that could have explained the observed instability of our preparations. In fact, the data obtained from these studies showed that the degree of preservation of metabolic enzyme activity after lyophilization was, in most cases, better than that obtained by conventional liquid storage of the cells in a blood-bank refrigerator at 4°C. We were forced to look elsewhere for an explanation of the instability of the preparations.

3. HEMOGLOBIN PRESERVATION

Our next series of studies focused on the stability of the hemoglobin present in the lyophilized cells. As we had noted earlier, the lyophilized preparations appeared to maintain high levels of oxyhemoglobin following lyophilization. Degradation of hemoglobin into its separate components of hemin and globin can lead to several disastrous consequences for the cells. These are reported extensively in the literature on red cell anemias, which arise due to genetic- or drug-induced alterations in hemoglobin function (Motulsky and Stamatoyannopoulos, 1967; Campwala and Desforges, 1981). In order to determine the extent of hemoglobin denaturation that may have occured during the lyophilization process, we examined preparations using methods of spectrophotometric as well as electron paramagnetic resonance (EPR) spectroscopy.

The data for the levels of oxyhemoglobin, methemoglobin and hemichrome present in these preparations were determined using a method described by Szebeni et al. (1984). The procedure requires the determination of the absorbance of the specimen at three wavelengths. In this way, it is possible to calculate the amount of each particular form of hemoglobin present in a preparation. Oxyhemoglobin is the form of hemoglobin that is capable of transporting oxygen to tissues in the body. It is in constant equilibrium in the body with its deoxygenated form. Under atmospheric conditions with high partial pressures of oxygen, the hemoglobin should exist primarily in this oxygenated form. Upon oxidation of the hemoglobin, methemoglobin is formed. This form of hemoglobin does not bind and transport oxygen. In the cell, methemoglobin formation is controlled by the

Table 6. Percentage hemoglobin species in reconstituted, lyophilized human red blood cells.

Hemoglobin species	Assay wavelength (nm)	Percent
Oxyhemoglobin	560	96.6 ± 2.0
Methemoglobin	577	2.9 ± 1.9
Hemichromes	630	Not detectable

Normal and fresh non-lyophilized human red blood cells contain 98–100% of oxyhemoglobin. Methemoglobin can be converted back to oxyhemoglobin and is present in the blood in concentrations of 1–2%. N=63.

methemoglobin reductase enzyme system. As we had previously shown, this system remained intact in the lyophilized cells. Hemichrome is a form of hemoglobin that is produced subsequent to formation of methemoglobin. This form of hemoglobin is a further altered structural form of hemoglobin that results from changes in the tertiary structure of the molecule after oxidation.

The data in Table 6 contains results from a direct spectrophotometric determination of the amounts of each species of hemoglobin present in the lyophilized preparations. These results indicated that the majority of hemoglobin obtained in the lyophilized cells was in the form of oxyhemoglobin. The results of this study were confirmed by tests of the oxygen releasing and binding properties of the cells. Analysis of the cells at various partial pressures of oxygen indicated that the cells showed normal oxygen release and binding properties when compared to control samples (Figure 6). The point at which 50% saturation of the hemoglobin with oxygen occured (known as the $P(50)$ value) was identical for lyophilized and non-lyophilized cells (26.3 ± 4.7 versus 27.0 ± 2.0 mm Hg). It did not appear as if the lyophilization process was severely altering the function of the oxyhemoglobin, which was present in these preparations. A key question remained relative to the amount of other forms of oxidized hemoglobin that may have been produced during lyophilization.

Hemichrome is a form of hemoglobin that can undergo further decomposition to form hemin and globin (Figure 7). These denatured forms of hemoglobin can be very destructive to the cell membrane (Liu et al., 1985; Kirschner-Zilber et al., 1982). Hemin, the porphyrin portion of the hemoglobin molecule, generally acts like a detergent, causing disruption and even dissolution of cell membranes. It can also function as an oxidant for cell proteins. Globin, the protein portion of hemoglobin, can also bind to the cell membrane and cause severe

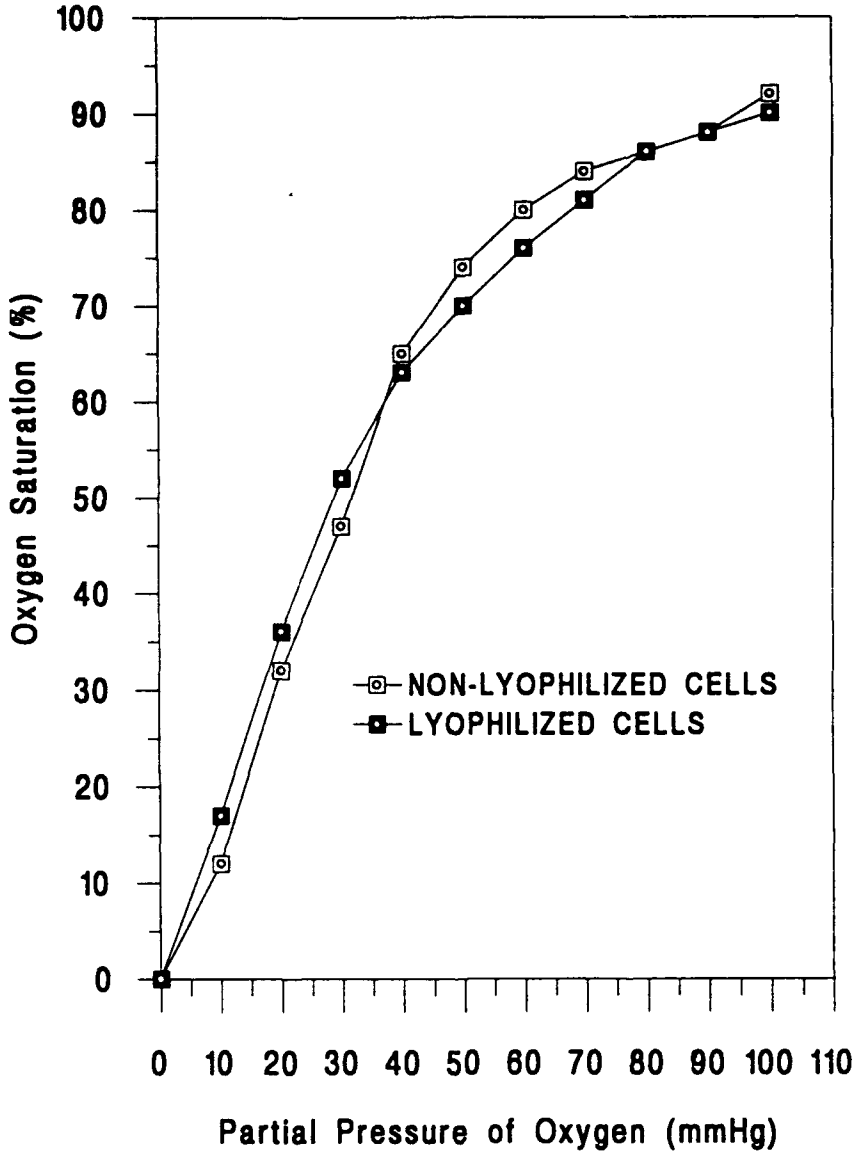


Figure 6. Oxygen equilibrium curves (which represent the affinity of hemoglobin for oxygen) of hemoglobin from lyophilized and non-lyophilized red blood cells. The curve for lyophilized red blood cells is sigmoidal and is similar to non-lyophilized red blood cells. This sigmoidicity is the result of cooperativity or heme–heme interaction, a phenomenon in which the oxygenation of one subunit of the hemoglobin tetramer alters the oxygen affinity of other subunits. The partial pressure at which the hemoglobin is 50% saturated with oxygen is 27 mm Hg for non-lyophilized red blood cells and 26 mm Hg for lyophilized cells.

alterations. We envisioned that if these agents were being produced either during or after lyophilization due to destabilization of the hemoglobin molecule, the observed alterations in cell stability could

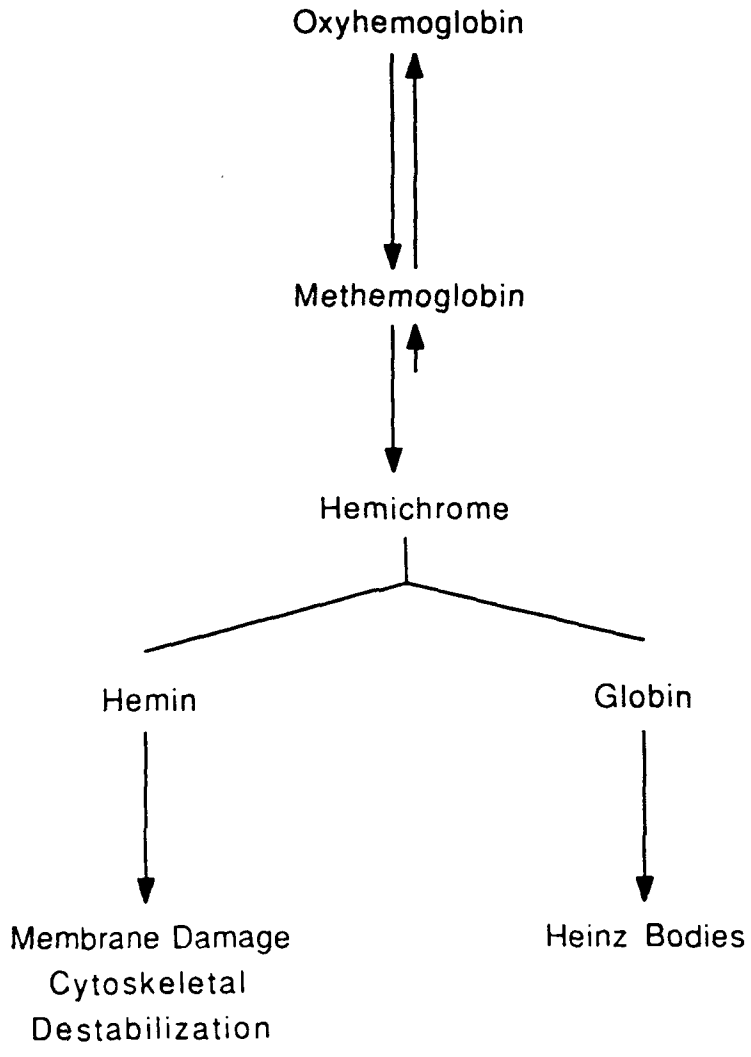


Figure 7. Schematic illustration of oxidative breakdown of hemoglobin to globin and hemin. In lyophilized red blood cells the level of hemin is about six times that of normal non-lyophilized red blood cells. This increase in hemin concentration in lyophilized red blood cells is not accompanied by extensive destabilization of the red cell structural integrity. Methemoglobin that is not reduced back to its functional state can gradually convert to a reversible hemichrome and then to irreversible hemichrome which aggregate to form a Heinz body.

be explained by their presence. As noted in Table 6, no hemichrome was detected by the spectrophotometric method that was employed. The sensitivity of this method is limited, however. This was an important consideration given that even millimolar amounts of hemin could induce significant levels of membrane damage. To determine the levels of hemin present in the samples, we, in collaboration with Dr. Matthew Platz at The Ohio State University, developed a

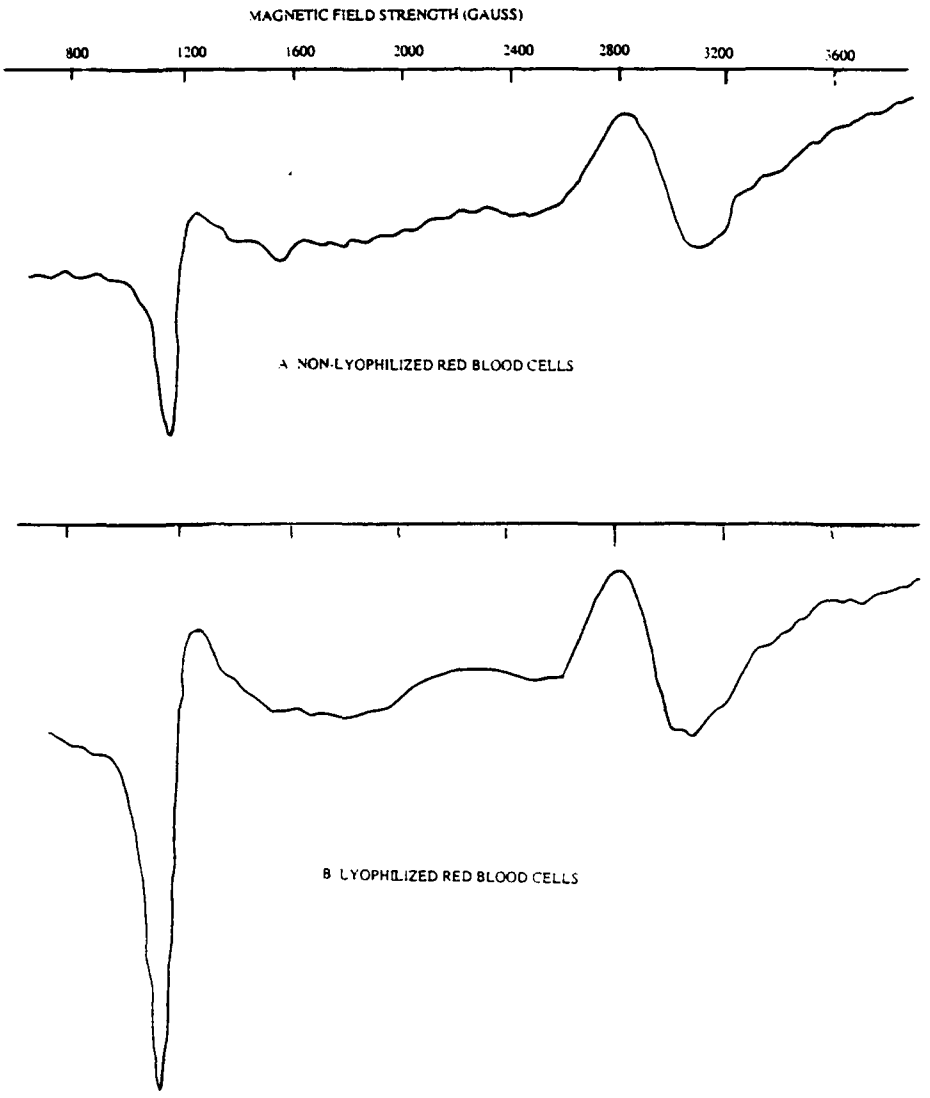


Figure 8. EPR spectroscopy of reconstituted, lyophilized human red blood cells. Hemin, a denatured oxidative breakdown product of hemoglobin, has a distinct absorbance at a magnetic field strength of 1100 gauss. Integration of the EPR spectra at the peak absorbance is used to calculate the amount of hemin present in lyophilized red blood cells. (A) Control, non-lyophilized red blood cells; (B): lyophilized blood cells.

technique using EPR spectroscopy. Hemichromes and hemin possess unique spin states that permit their detection by means of EPR spectroscopy. This technique had been used previously to determine the levels of hemin or hemichromes present in pathological red cells (Peisach et al., 1971). Figure 8 shows typical spectra obtained for samples in the region of 1100 Gauss where hemin is known to absorb (Rachmilewitz et al., 1971, 1974). The signal intensity observed could be directly correlated with the amount of hemin present in a

Table 7. Levels of hemin and lipid hydroperoxide in reconstituted, lyophilized human red blood cells and control non-lyophilized cells.

Samples	Hemin detected by EPR (mM)	Lipid hydroperoxide (nmol/mg lipid)
Control	0.154 ± 0.152	0.78
Lyophilized	0.727 ± 0.152	1.09

calibration sample. Data from these studies are summarized in Table 7. The level of hemin detected in control samples using this method was 0.154±0.152 mM. In lyophilized samples, the amount of hemin detected was 0.727±0.152 mM. Although this level of hemin is higher than in control samples, this amount did not produce excessively elevated levels of lipid peroxides (Table 7). This latter work was carried out in collaboration with Dr. Robert Hebble at the University of Minnesota.

It appeared from our analysis of hemoglobin structure and function in lyophilized samples that a mechanism involving or requiring denatured hemoglobin was not likely to explain the magnitude of cell instability that was being observed in our samples. We decided at this point to turn our attentions to the red cell cytoskeleton.

4. CYTOSKELETAL STUDIES

The red cell cytoskeleton is comprised of a series of proteins including actin, spectrin, and band 4.1. These proteins form a complex mesh network beneath the cell membrane that provides support to the red cell membrane (Lux, 1979; Mohandas et al., 1983). This cytoskeletal structure is both flexible and supportive and is of utmost importance in maintaining the integrity of the red cell deformability characteristics. We had previously observed that the red cells obtained after lyophilization were not deformable. This behavior was evident when the cells were examined using the techniques of ektacytometry and filtration measurements through miroporous filters. The method of ektacytometry was originally developed by Dr. Marcel Bessis in France and Dr. Narla Mohandas (Groner et al., 1980; Mohandas et al., 1980, 1981).

In collaboration with Dr. Mohandas at UC Berkeley, we evaluated the deformability of the red cells using the ektacytometer. This device functions by placing the cells under a shear stress induced by

Table 8. Rheological properties of reconstituted, lyophilized human red blood cells.

Sample	Maximum cell deformation at shear stress of 320 dyn/cm²	Cell transit time through 5 μm filters at 3cm H_2O
Control	0.560 ± 0.04 *n* = 23	3.12 ,± 0.30 ms *n* = 14
Lyophilized	0.350 ± 0.03 *n* = 3	4.64 ± 1.89 ms *n* = 15

Data represent the mean ± SD. Transit time of red blood cells through 5 μm pore diameter filters were measured with a cell transit time analyzer at a driving pressure of 3 cm H_2O at 22°C. The covariance of cell transit time measurements for normal non-lyophilized human red blood cells is 21.7 ± 1.34% compared with 176.2 ± 98.7% for reconstituted, lyophilized cells. The convariance of the cell transit times is a measure of the heterogeneity of the cell population.

the medium viscosity in which they are suspended. This solution is mixed at various rates, allowing the shear stress induced on the cells to vary. A shear stress corresponding to 162–200 dyn/cm² (200 rpm in the cylindrical viscometer) normally induces maximal deformation in a manner consistent with shear stresses that are induced as cells pass through the microvasculature. By measuring the degree of ellipticity obtained from a diffraction pattern for cells undergoing this stress, it is possible to determine the amount of deformability that the cells can tolerate.

Table 8 and Figure 9 present data obtained for control and lyophilized samples. The data in Table 8 clearly indicated that the deformability and filterability of lyophilized cells were severely compromised following lyophilization. In the best case, values of deformability that were only 60% of the control value could be obtained. The reduction in deformability was dramatically confirmed by examination of the filterability of lyophilized red cells through 5 μm pore filters using the cell transit time analyzer as described by Koutsouris et al. (1988). In these cases, the mean transit times of lyophilized cells (i.e. the mean time required for cells to traverse a pore) was greatly increased over that of control cells and the covariance of the measurement was about five times that of normal. The levels of reduced filtration and deformation observed in lyophilized samples were much higher than those that are observed even in cases of severe membrane alteration that occur in clinical cases of spherocytosis and sickle cell disease (Clark et al., 1983). Alterations of this magnitude are often induced by changes in the

composition or organization of the membrane cytoskeleton. For this reason, we felt that alteration in this structure as a result of lyophilization was a likely candidate for the instability we had observed.

We decided to study the cytoskeleton using a technique developed by Dr. David Liu and Dr. Jiri Palek at Tufts University. This technique allowed researchers to spread the cytoskeletons of red cell ghosts and peel away the membrane cover to make it possible to examine via electron microscopy the structural integrity of the cytoskeleton. In this way, it is possible to examine the interaction of proteins with one another to determine if the appropriate protein–protein interactions are being maintained. Dr. Liu and Dr. Palek had successfully applied this technique in the study of red cells of patients with hereditary anemias resulting as a consequence of genetic defects that produced decreased levels of spectrin or actin in the cells (Liu et al., 1990).

In collaboration with Dr. Liu and Dr. Palek, we prepared samples of lyophilized red cells and examined their cytoskeletal structure using gel electrophoresis and the electron microscopic methods. We were intrigued by the results. The data listed in Table 9 clearly show that gel analysis of the cytoskeletal proteins failed to indicate any deletion or significant reduction in the amounts of the individual proteins. More interesting was the comparable levels of spectrin dimers present in the control and lyophilized samples. Lyophilization of the cells did not appear to alter the protein interactions at this level. The presence of the individual proteins was confirmed with immunoblotting of the gels derived from the lyophilized cell preparations. This level of preservation was also confirmed when electron micrographs of the spread cytoskeletons were examined (Figure 10). The spread cytoskeletons of the lyophilized cells showed comparable levels and extents of the classical hexagonal arrays corresponding to spectrin–actin–band 4.1 interactions present in normal, untreated samples. It did not appear from these studies that there was any alteration in the membrane cytoskeleton that was indicative of a pathological state.

Upon examination of the electron micrographs of lyophilized red cell samples, however, another distinct lesion did appear. This lesion was not associated with the cytoskeleton, but rather appeared in images in which the cell membranes were depicted (Figure 11). The lesion appeared to be manifested by a disruption or discontinuity in

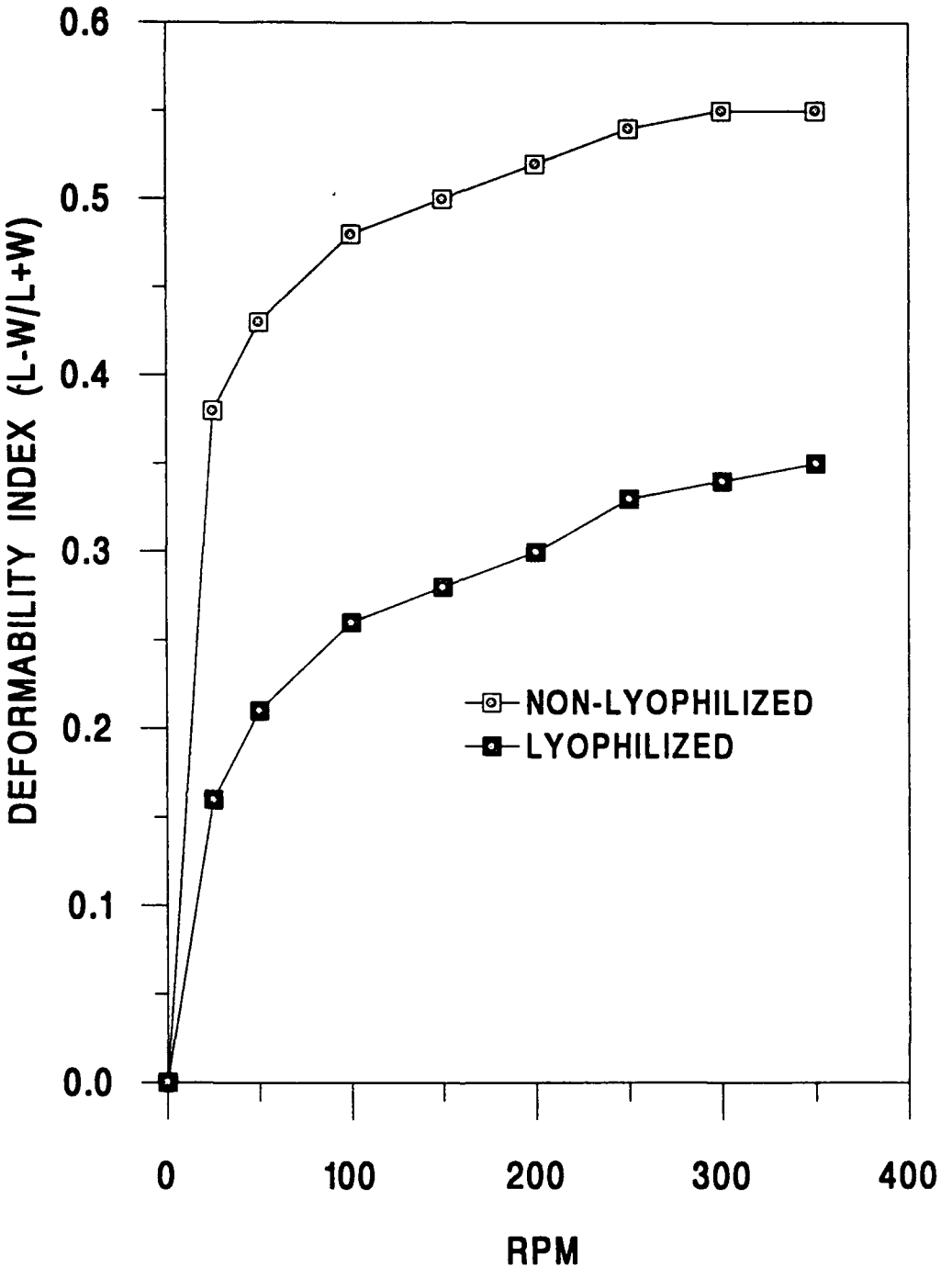


Figure 9. Measurement of red blood cell deformation as a function of applied shear stress (varies from 0 to 320 dyn/cm^2) expressed as rpm (0-400 rpm) of rotating inner cylinder of concentric cylinder viscometer. The deformation index (DI) is defined as $(L-W)/(L+W)$ and represents the ellipticity of the ektacytometer diffraction patterns calculated by measuring the light intensity of the laser diffraction pattern at two points, the major axis (L) and the minor axis (W) dimensions.

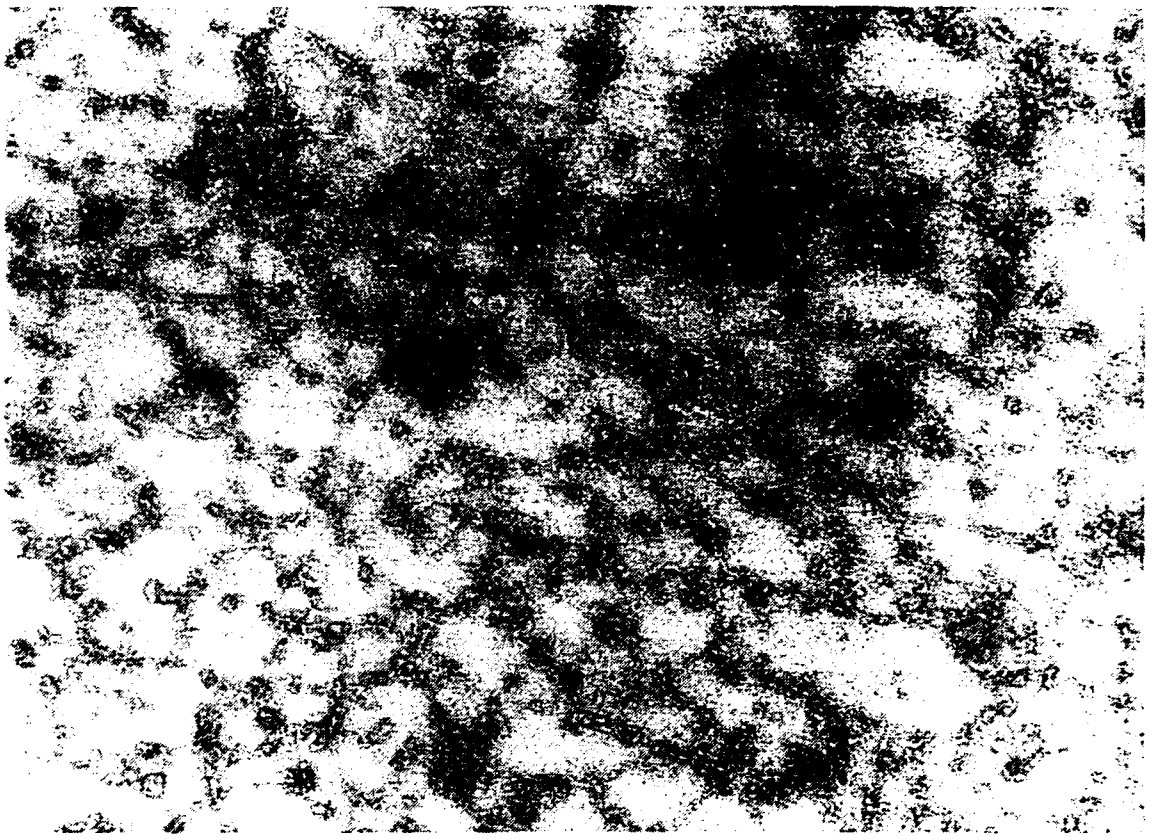
Table 9 PAGE analysis of reconstituted, lyophilized red blood cell membranes and fresh control non-lyophilized RBC membrane.

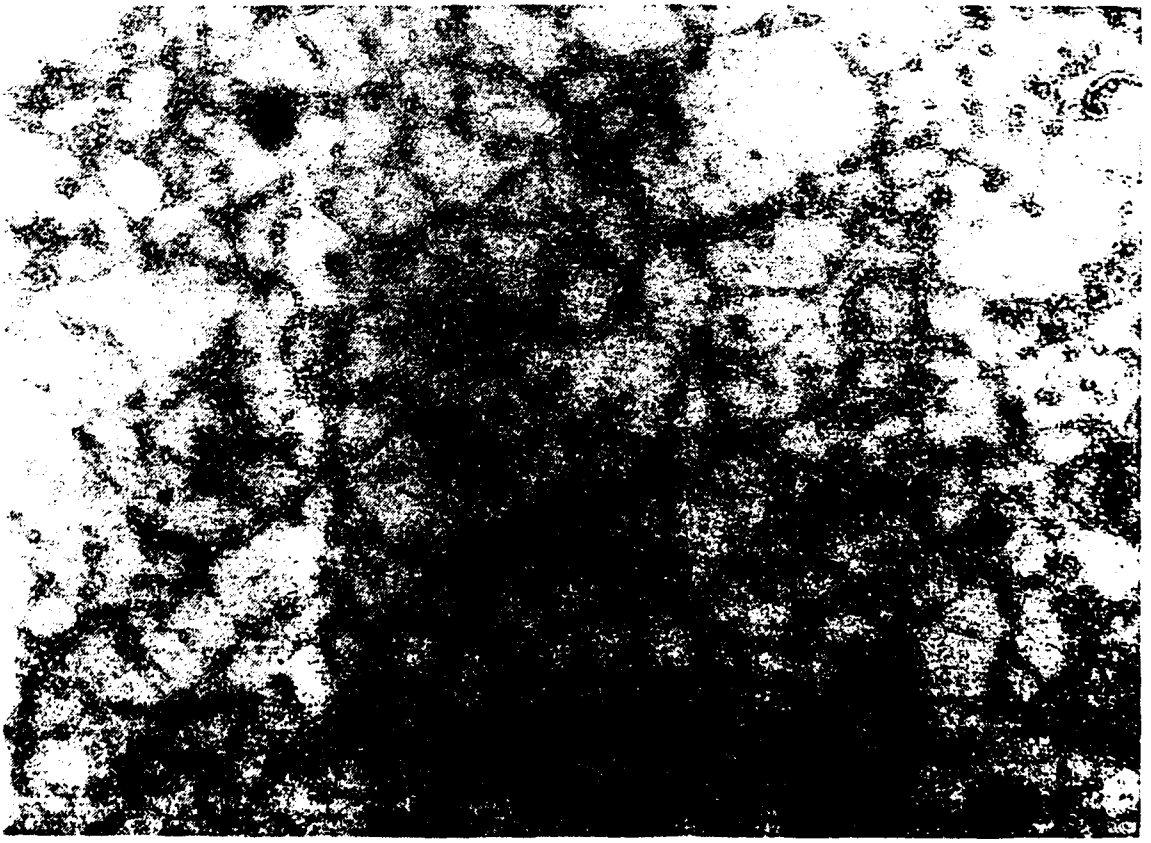
Test	Control RBC	Lyophilized RBC
Gradient Fairbanks gel		
αSp/βSp	1.15	1.06
Sp/band 3	1.05	0.99
2.1/band 3	0.16	0.11
4.1 + 4.2/band 3	0.35	0.36
Laemmli gel		
4.1a/4.1b	1.5	1.6
4.1/4.2	1.09	1.08
Spectrin dimers	6%	4%
Immunoblots		
anti-spectrin	normal	normal
anti-2.1	normal	normal
anti-4.1	normal	normal

The membrane of the human red blood cells is reinforced along its cytoplasmic surface by a two-dimensional network of peripheral proteins that are anchored to the bilayer by a number of integral proteins. The cytoskeleton is composed of three principal components, spectrin, actin and protein 4.1. Spectrin (α and β) accounts for 75% of the total skeletal mass. The physical linkage of membrane skeleton to the lipid bilayer is accomplished by ankyrin (band 2.1), which simultaneously interacts with spectrin and band 3.0. The second linkage is provided by band 4.1, which comprises about 5% of the red cell skeletal mass and migrates as a closely spaced doublet 4.1a and 4.1b in sodium dodecyl sulfate–polacrylamide gel electrophoresis (SDS–PAGE) with the discontinuous, Laemmli buffer system.

the lipid bilayer structure in several regions of the cell membrane. The control samples showed this lesion in less than 1% of the total number of cells in a given field whereas as many as 50–60% of the lyophilized cells possessed some form of a membrane lesion. This pointed to some distinct alteration occuring in the cell membranes after lyophilization.

Figure 10. Electron micrographs of cytoskeletal proteins from fresh non-lyophilized red blood cells (A) and reconstituted, lyophilized red blood cells (B). Pictures show the arrangements of the spectrin and actin and associated cytoskeletal proteins into a network for stabilizing the red cell membrane and controlling cell shape and deformation. The arrangement of the membrane cytoskeleton in lyophilized red blood cells is identical to non-lyophilized red blood cells.





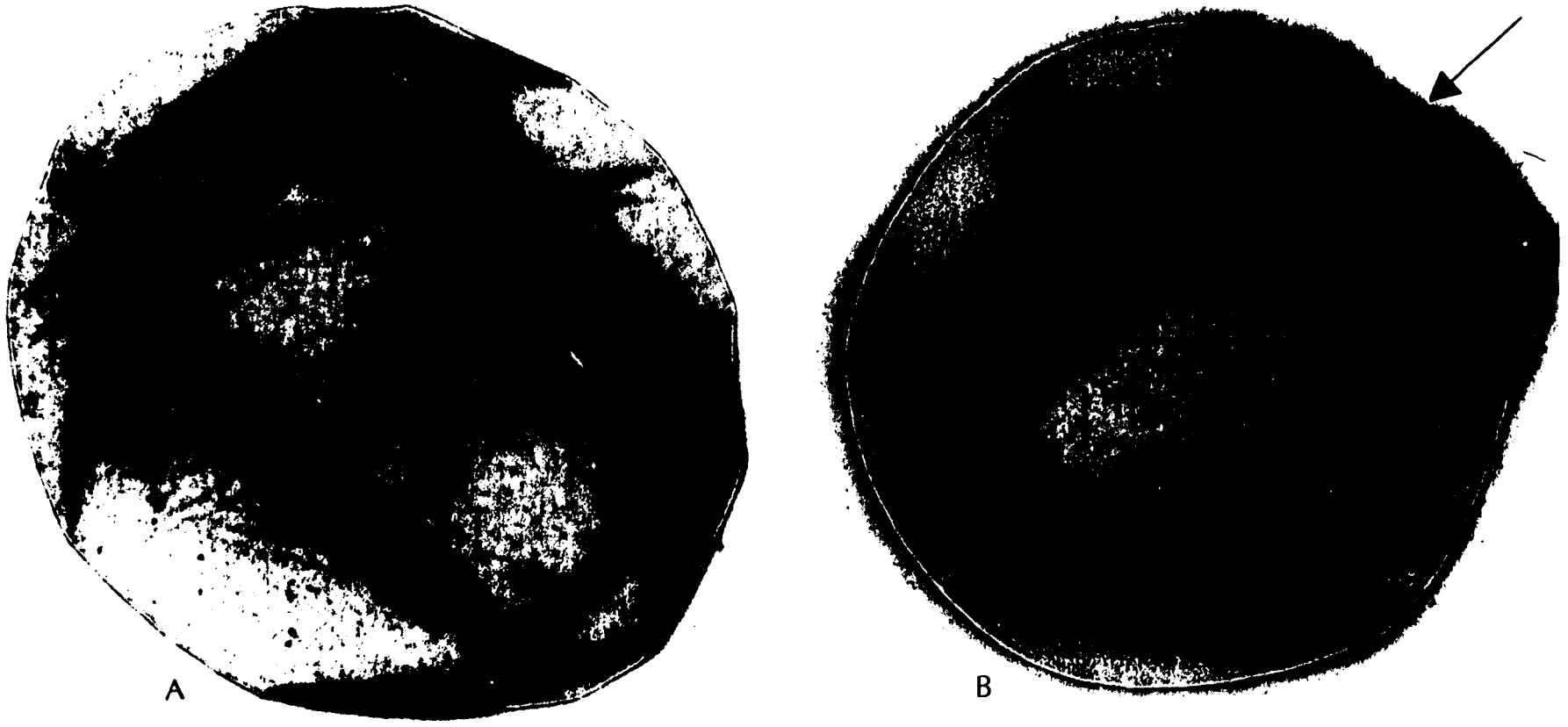


Figure 11. Electron micrographs of flattened red cell ghosts from non-lyophilized (A) and lyophilized red blood cells (B). Arrows indicate areas of discontinuity in the membrane lipid bilayer in lyophilized cells. Control non-lyophilized cells did not show any damage to membrane phospholipid bilayer.

5. MEMBRANE STUDIES

Meryman's original description of his own work in this field involved what he perceived to be an artefact created by the surface-tension properties of polymers replacing what was an essentially absent membrane. This appeared to be true in the case of rat and guinea pig cells, but very different for human samples. Our electron microscope (EM) images of lyophilized red cells showed what appeared to be a membrane with areas of lesions or structural disruptions. Although the membrane was compromised, it was still present. These results were confirmed by an examination of the phospholipid content of the cells. Samples of lyophilized red cells and control red cells were extracted using chloroform and methanol. The resulting samples were assayed for the total amount of phosphate present in the phospholipid extract using the Bartlett assay (Bartlett, 1958). The data indicated that the amount of lipid present in the control samples was 13.09 mg/ml RBC whereas lyophilized samples possessed only 10.6 ± 0.98 mg/ml RBC. There appeared to be an overall decrease of membrane surface area.

The consequences of this membrane deletion in lyophilized samples were manifested in their ability to bind significantly larger amounts of merocyanine and take up increased levels of fluorescein diacetate than control samples (Table 10). The increased binding of

Table 10. Fluorescence intensity profiles of human red blood cells labeled with merocyanine 540 (MC540) and fluorescein diacetate (FDA).

Sample	Fluorescence intensity (arbitrary units)	
	MC540	FDA
Control	11.1 ± 2.5 $n = 14$	95 (90-100) $n = 2$
Lyophilized	14.7 ± 3.3 $n = 23$	157.9 ± 41.2 $n = 6$

Data represents the mean ± sd. Packing of red cell membrane lipids was assessed with the fluorescent lipophilic dye, merocyanine. Merocyanine is normally excluded from tightly packed lipid bilayers, but readily binds to those that are loosely packed. Total fluorescence intensity (expressed in arbitrary units) is elevated in lyophilized cells when compared to fresh non-lyophilized cells. Fluorescein diacetate does not penetrate intact human red blood cell membranes. However, damaged cells or cells with leaky membranes (i.e. osmotically unstable) readily take up FDA.

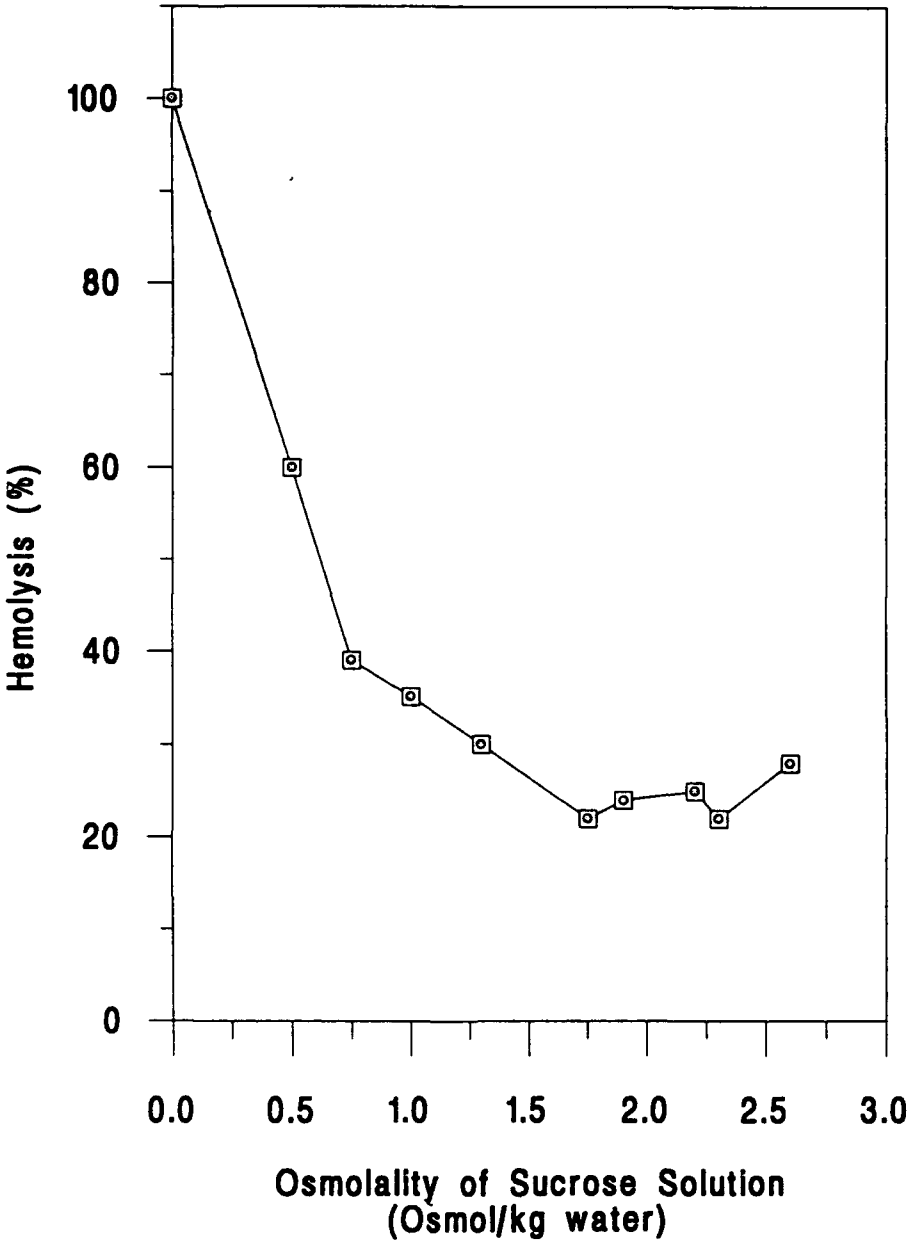


Figure 12. Cell hemolysis as a function of suspending medium osmolality. The main factor affecting the volume regulation of normal red blood cells is the surface area to volume ratio. Loss of cell membrane or a decrease in surface area will severely compromise the ability of red blood cells to regulate their volume.

these dyes to red cells is a direct indication of severe perturbation of the lipid structure in the cells (Williamson et al., 1983; Schlegel et al., 1980). It appeared at last that we had an explanation for our observed reduction in cell stability in isotonic solutions without polymer substituents.

6. GENERAL HYPOTHESES

The loss of membrane lipid via a deletion of membrane substance either as a direct result or a consequence of lyophilization would be expected to have severe effects on the cells. Our data on these lyophilized preparations indicated that the cells were still intact

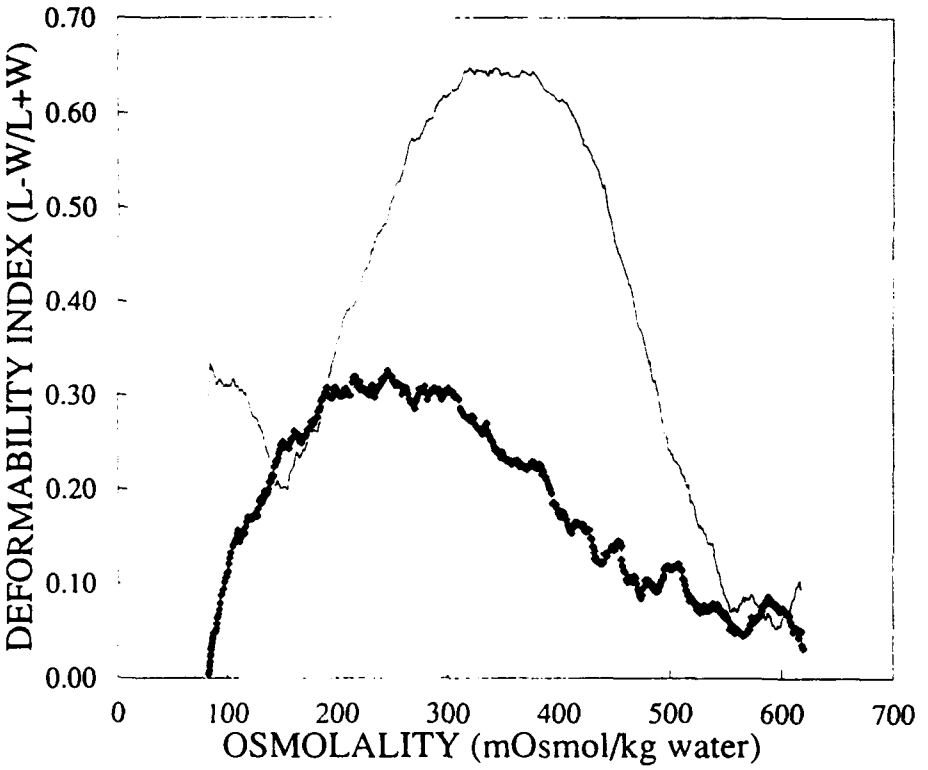


Figure 13. Osmotic deformability profile for rehydrated, lyophilized red blood cells (-◆-), and control (non-lyophilized) red blood cells (——). Osmoscan of freshly drawn red blood cells is included for comparison with experimental sample. The osmotic deformability profiles indicate the responses of the red blood cells to different osmotic stresses at a fixed shear stress of 230 dyn/cm^2. It allows for a detailed analysis of the various factors that determine the rheological properties of red blood cells. The essential features of these curves are: (a) hypotonic response of the red blood cells is determined primarily by the surface area to volume ratio. Cells with a high ratio show a left shift (i.e. increased osmotic resistance); (b) hypertonic response is determined by the internal viscosity and membrane rigidity; (c) isotonic response is determined by all the various factors affecting red cell deformability (surface area to volume ratio, membrane rigidity, internal viscosity). In lyophilized red blood cells, the hypotonic response is completely absent and indicates membrane instability due to alteration in surface area to volume ratio.

following lyophilization, but had undergone significant reductions in their ability to cope with normal isotonic situations. Studies of the ability of the cells to respond osmotically indicated that the cells were capable of this behavior only under hypertonic conditions. Figure 12 depicts the level of cell hemolysis observed as a function of solution osmolarity. At levels of osmolarity that are in excess of 800 mOsm,

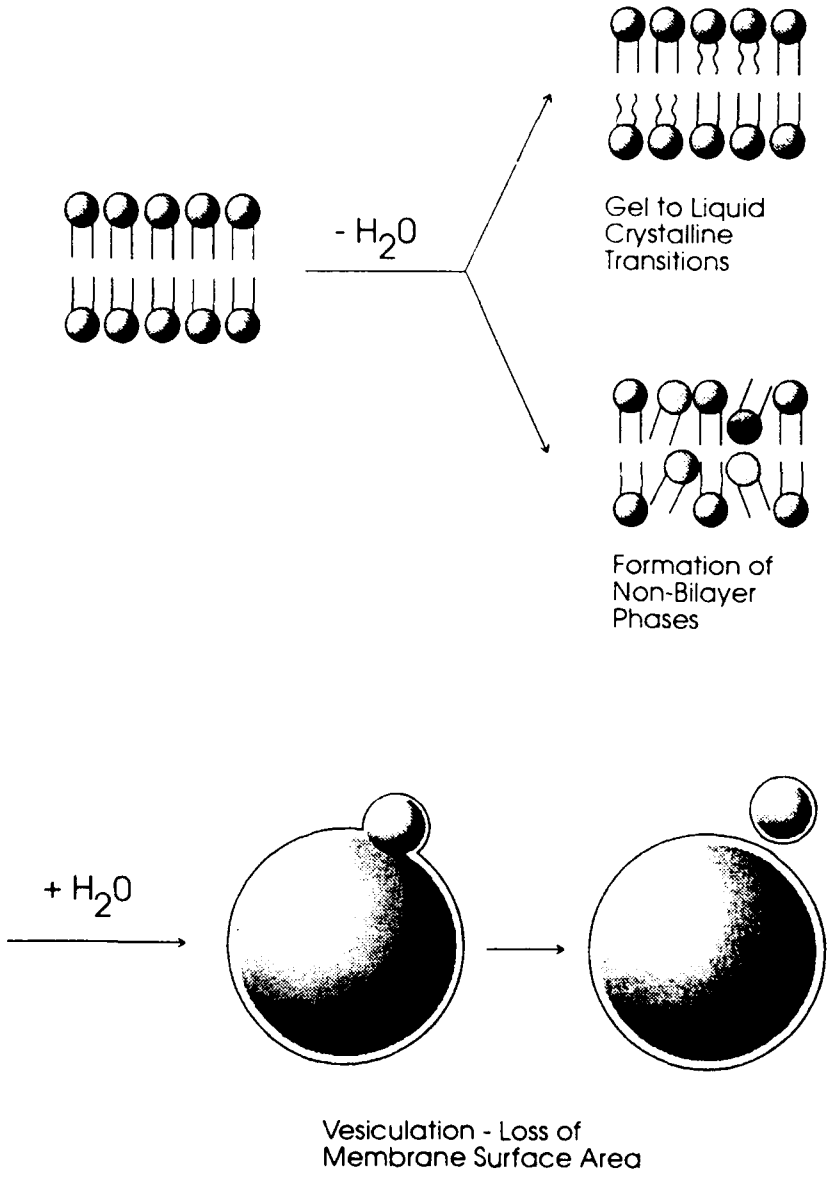


Figure 14. Theoretical consideration of the mechanisms responsible for cell damage during processing, lyophilization, and reconstitution of lyophilized, human red blood cells. Removal of water leads to changes in lipid phases. These changes promote vesiculation and loss of membrane integrity.

the lyophilized cells appear to be stable. When the osmolarity was reduced toward normal isotonic conditions, however, the amount of hemolysis proceeded to completion. It was almost as if the cells were no longer balanced against isotonic media, but had shifted to being balanced against solutions of much greater osmolarity. The deformability profiles of the cells obtained with the ektacytometer as a function of osmolarity confirmed this feature (Figure 13). In this case, the cells were not able to achieve maximum deformation under isotonic conditions. This behavior was a direct indication of severe alterations in the cell surface area to volume ratio (Clark et al., 1983). Loss of membrane surface area without a proportionate loss in cell volume requirements can produce each of these effects. The situation is analogous to one in which a balloon with a given surface area and given volume is deflated and cut so as to remove a large segment. If the balloon is resealed around the missing piece and blown up to the same volume that it possessed prior to deletion of some of its material, it can explode or be on the verge of exploding. The balloon simply does not have a sufficient amount of surface area to accommodate its normal volume requirements. This effect appeared to be happening in the case of our lyophilized red cell preparations. The main question was why this was happening. We arrived at two possible mechanistic explanations.

When lipid vesicles are subjected to dehydration, they undergo several changes in the lipid structure. These effects were well documented in studies by Crowe et al. (1988) and Goodrich et al. (1990, 1991) using liposome preparations. The changes that occur include formation of hexagonal phase and adjoining regions of gel and liquid crystalline phase lipids. The formation of these regions leads to physical destabilization of the membrane, an event that is believed to foster fusion, aggregation, and leakage of the vesicles (Siegel et al., 1989). If this same behavior were to occur with the red cells, the results would be similar as in the case of the liposomes. In this case, membrane lipid phase changes could be responsible for leakage of the cells and loss of membrane surface area by direct vesiculation of the membrane. Such behavior would explain the reductions in deformability and osmotic stability as described previously and would provide a direct explanation for the detected loss in membrane phospholipid for the reconstituted cells. Reports in the literature for red cells subjected to long-term storage in the hydrated state indicate that such vesiculation processes do occur with aging and lead to a reduction in the deformability and filterability of

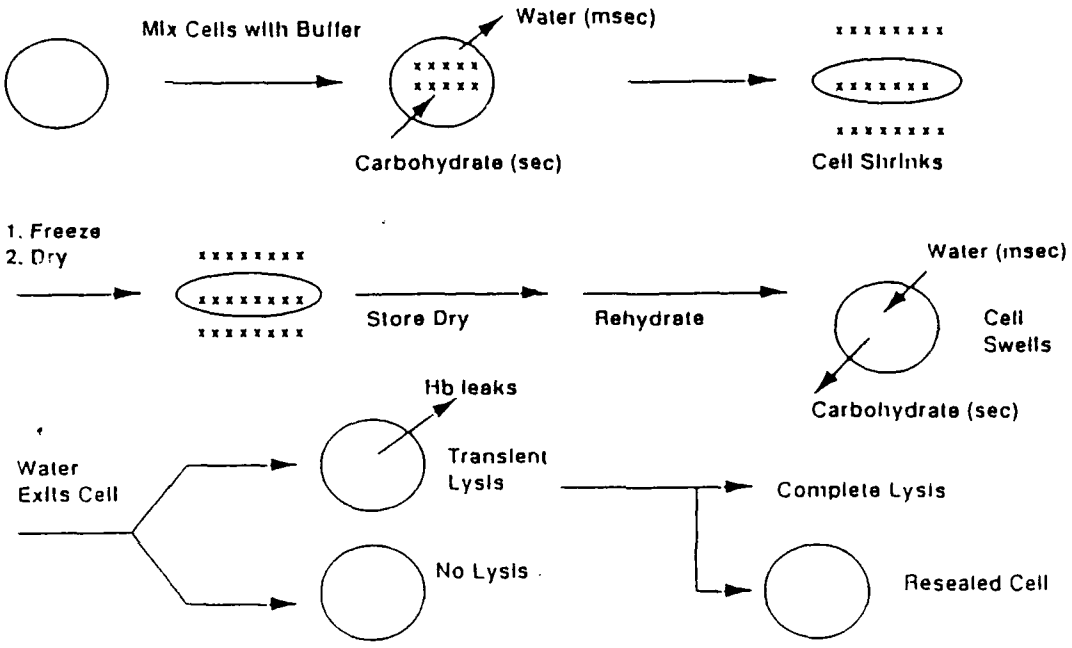


Figure 15. Alternative hypothetical mechanism for cell instability. Removal of water creates 'rehydration paradox', which can cause cell lysis and resealing during rehydration.

the cells (Greenwalt et al., 1991). The mechanism proposed for this behavior in the lyophilized red cell is outlined in Figure 14.

Another possible mechanism for the observed behavior of the lyophilized cells was the loss of membrane surface area as a result of the stresses occurring during rehydration. Figure 15 depicts a scheme for the entire lyophilization and rehydration process. In this scheme, samples are normally placed in a medium containing a permeating carbohydrate and a polymer. When the samples are lyophilized, the water is removed, causing the concentration of components inside and outside the cells to increase toward infinity. When the solid is rehydrated, there is a transient gradient for water to re-enter the cells. The size of this gradient with the first drop of water is enormous and decreases as the solid is dissolved. Since the outside of the cell is rehydrated first, the gradient will always go from outside to inside for the water flow. This behavior could have profound consequences for the cells. If the water enters too rapidly, the cells could swell and lyse before they have the opportunity to regulate their volume. This lysis event may be accompanied by a resealing event with a loss of membrane vesicles in the process. The resealed cell would then possess an altered surface area to volume ratio relative to the starting cell.

Each of these mechanisms could produce the lyophilized cells we had observed. Preventing this damage appeared to be paramount in obtaining a cell that could be classified as completely viable and more importantly serve as a suitable material for use in red-cell transfusions. Both mechanisms are very different in proposing what goes on, but both have the same ultimate end point. In addition, both mechanisms have another feature in common. Both are products of the lyophilized samples having extremely low moisture contents. In the case of a membrane alteration due to lipid phase changes, it is the removal of water from the lipid head groups that is required for such alterations to occur (Bush et al., 1980). In the case of the lysis-resealing model induced by osmotic gradients, it is the removal of water in the first place that creates the rehydration paradox. Regardless of the extent to which either mechanism may have been operating, the key to both appeared to have been in developing a system in which the cells could be dried to higher water contents while still exhibiting the stability and storage properties that make a lyophilized product so highly desirable.

7. CURRENT STATUS OF DEVELOPMENT

In the summer of 1991, we discovered just such a system for carrying out this feat. The procedure relies on the application of a set of principles in chemistry known as glass-transition theory. This model for the behavior of polymeric systems has been well-characterized in the field of materials science (Nicholson, 1991). In recent years, it has found utility in applications in the fields of food science and cryobiology associated with protein preservation (Levine and Slade, 1988; Franks, 1991).

Simply stated, glass-transition theory can be used to describe systems that exist in states of extremely high viscosities. It can be applied to solutions where viscosities reach levels at which molecular motion within the solution is reduced so as to become imperceptible on real-time scales. During the freezing process, water partitions into the ice phase leaving behind a solution of higher concentration than the starting solution. This concentrated solution contains all of the materials present at the beginning of the freezing process. It contains

all of the remaining unfrozen water, the biological agent, and the components used to make up the cryoprotective formulation. As freezing continues, this material becomes even more concentrated as more and more water begins to partition into the ice phase. Eventually, this concentration effect becomes self-defeating for the process of ice formation. As the solution becomes more concentrated, the rate of molecular motion and diffusion processes become retarded. The rate of ice formation also decreases. At a characteristic temperature known as the glass-transition temperature, the rate of ice formation or other diffusion-dependent chemical processes become slowed to the point of being imperceptible on a real-time scale. Since reactions that degrade or lead to breakdown of biological materials are dependent on chemical reactions, which are in turn controlled by diffusion or molecular motion, the cells or other biological material suspended in this matrix are in a state of stasis. The point at which this state is reached is dependent on the nature of the components comprising the starting solution.

The components we had used in preparing the lyophilized cells consisted of carbohydrates and polymers. Empirically, we had found that polymers of higher molecular weight afforded better protection during drying than the low molecular weight polymers. We had never been able to adequately explain the reason for this behavior. Glass-transition theory affords a direct explanation of this phenomenona due to the fact that molecules with low molecular weight normally exhibit low glass-transition temperatures whereas higher molecular weight compounds exhibit high glass-transition temperatures. It is this higher glass-transition temperature for the solution that affords greater stability for the sample during drying and subsequent storage. Each component used in the solution posseses its own glass-transition temperature. These transition temperatures can be determined directly using the techniques of differential scanning calorimetry or differential mechanical analysis or other procedures. Mixtures possess glass-transition temperatures that are determined by the weight ratios of the components that are present in the solutions (Nicholson, 1991). It is therefore possible to balance the low glass-transition temperature of one component by mixing it with a substance possessing a higher glass-transition temperature. It is also possible to predict the glass-transition temperature of the solution by using the weighted averages of each of the components comprising the solution and their individual glass-transition temperatures (see Table 11).

Table 11. Measured glass-transition temperatures values reported in the literature.

	Cryopharm	Literature
Glucose	−44.8	−43
HES	−14.4	−13.5
PVP	−19.2	−21.5
Mannose	−44.4	−41
Xylose	−51	−48
Trehalose	−30.0	−29.5
Sucrose	−32.5	−32.0

In this manner, glass-transition theory can be used to predict the boundary between stability and instability for a frozen or dried material. Below the glass-transition temperature for the mixture, the sample would be suspended in a matrix in which chemical or biological processes were imperceptibly slow. Above the glass-transition temperature, however, the same sample would be suspended in a solution of extremely high concentrations of components capable of diffusion and molecular motion. Under these conditions, rapid degradation of the material could occur. Because of the high concentration of this phase, these reactions would occur at exponential rates, being governed by Williams–Landel–Ferry kinetics, rather than Arrhenius kinetics, which applies for dilute suspensions of materials (Williams et al., 1955). This feature is of extreme importance when considering the freezing and drying profile of the material intended to be lyophilized.

The glass-transition temperature represents the demarcation line between stability and instability for a frozen material. It also represents the same demarcation line for a material that is freeze-dried. As the state diagram in Figure 16 illustrates, the glass-transition temperature varies as a function of the amount of water that is present. Water acts as a plasticizer for the glassy state. With drying, water is removed from the ice phase during primary drying and then is sublimed from the glassy phase during secondary drying. Normally, drying must be carried out at temperatures above −40°C in order to be practically accomplished. Below this temperature, the vapor pressure of water is so low that the drying process proceeds at an extremely low rate, if at all (Figure 17). This requirement forces a need to have stability for the preparation in question at temperatures above -40°C and to maintain this stability during drying. For example,

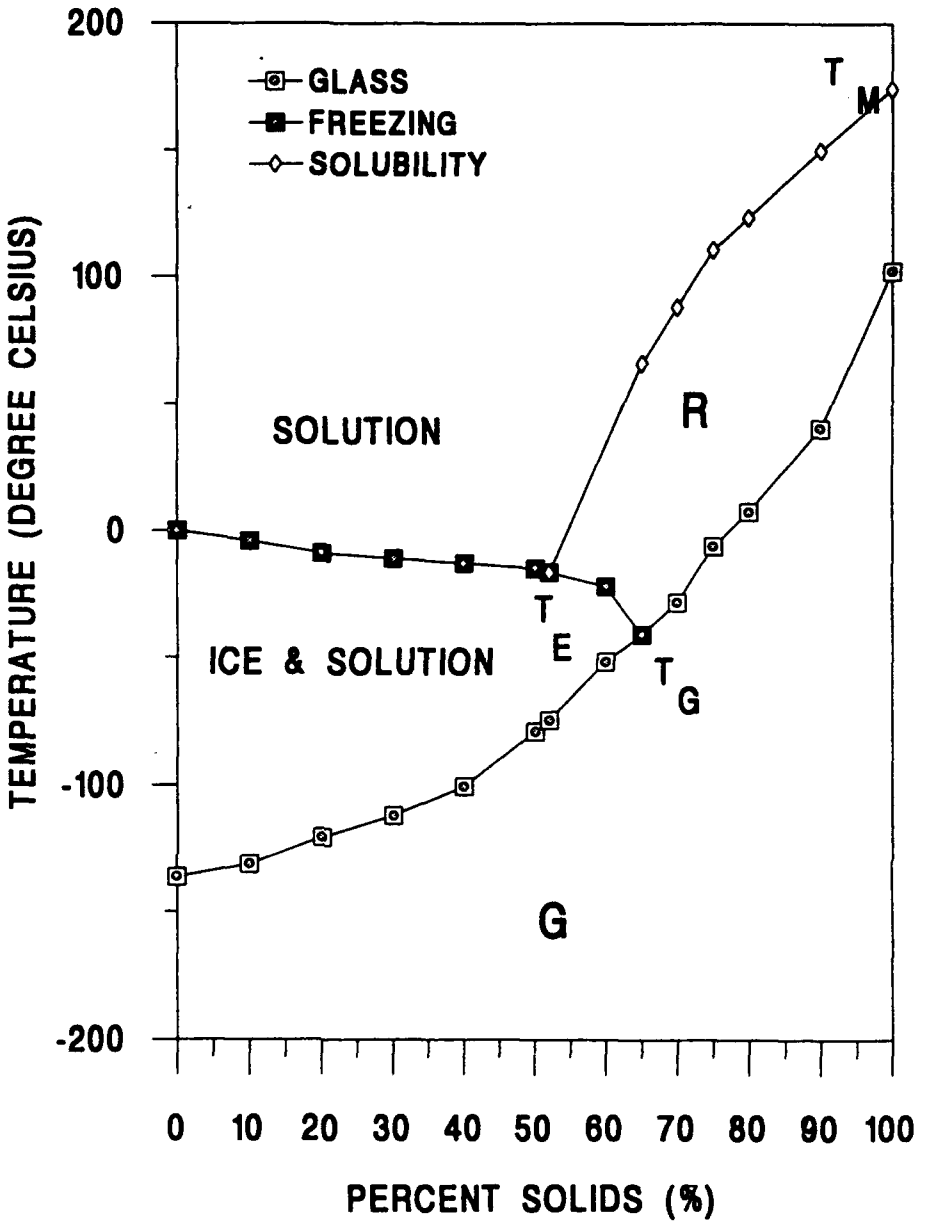


Figure 16. Theoretical state diagram for an aqueous solution. The diagram shows the boundaries between the solution (SOL), rubber (R), glass (G), and ice plus solution phases (ICE & SOL). The eutectic point for the solution is designated by T_E. The temperature at which the glass and freezing curves meet is designated as T_G. The melting point for the sample is designated as T_M. As freezing occurs, the concentration of the solutes in the remaining unfrozen water increases (% solids increases) until a point is reached at which individual components can crystallize out of solution (the eutectic point). If the viscosity is high enough, this crystallization of individual components is prevented and the sample continues to freeze concentrate until reaching the glass-transition temperature. The glass phase is characterized by extremely high viscosities. These high viscosities inhibit further ice growth and

Table 12. Calculated and observed T_g' values of mixtures

	Observed	Predicted
5% Glucose, 20% Dextran-40	−21.2	−22.4
5% Glucose, 10% Lactose, 10% Dextran-40	−30.2	−29.6
5% Glucose, 10% BSA, 20% Dextran-40	−20.5	−21.2
10% Glucose, 10% Lactose, 20% PVP-40	−32.7	−33.6
5% Glucose, 10% Trehalose, 20% PVP-40	−30.7	−31.2
18% Glucose, 8% BSA, 15% HES-500	−35.3	−33.2

secondary drying is normally carried out at a higher temperature than primary drying due to the need to desorb water directly from the glassy state. As more water is removed, the glass-transition temperature increases, making it feasible to increase the drying temperature and hence driving force for drying even further. At no time, however, should the drying temperature exceed the glass-transition temperature as this will impart mobility to the glassy matrix and lead to degradation of the product suspended in the glass. In this manner, glass-transition theory applied to these systems provides a series of guidelines for formulating the cryoprotective solution and performing the drying steps.

When we applied these principles to the solution we had developed for lyophilizing the red cells, we were amazed at the level of predictive capacity demonstrated by glass-transition theory. Table 12 contains a list of components and mixtures of components and the predicted and observed glass-transition temperatures obtained using an empirically derived formula. As can be seen, there was a very good correlation between the predicted and observed values. Using this approach, we also examined the behavior of red cell samples suspended in these solutions. Figure 18 shows the level of recovery of intact cells obtained following freezing of the cells and storage for brief intervals at various temperatures. The amount of hemolysis

chemical processes that may occur among the various components present in the unfrozen fraction. This delineates the region between stability and instability, since above this temperature, flow can occur in the unfrozen fraction. Drying leads to an increase in T_C by removing the plasticizing effect of water. This permits storage at high temperatures while maintaining the glassy state.

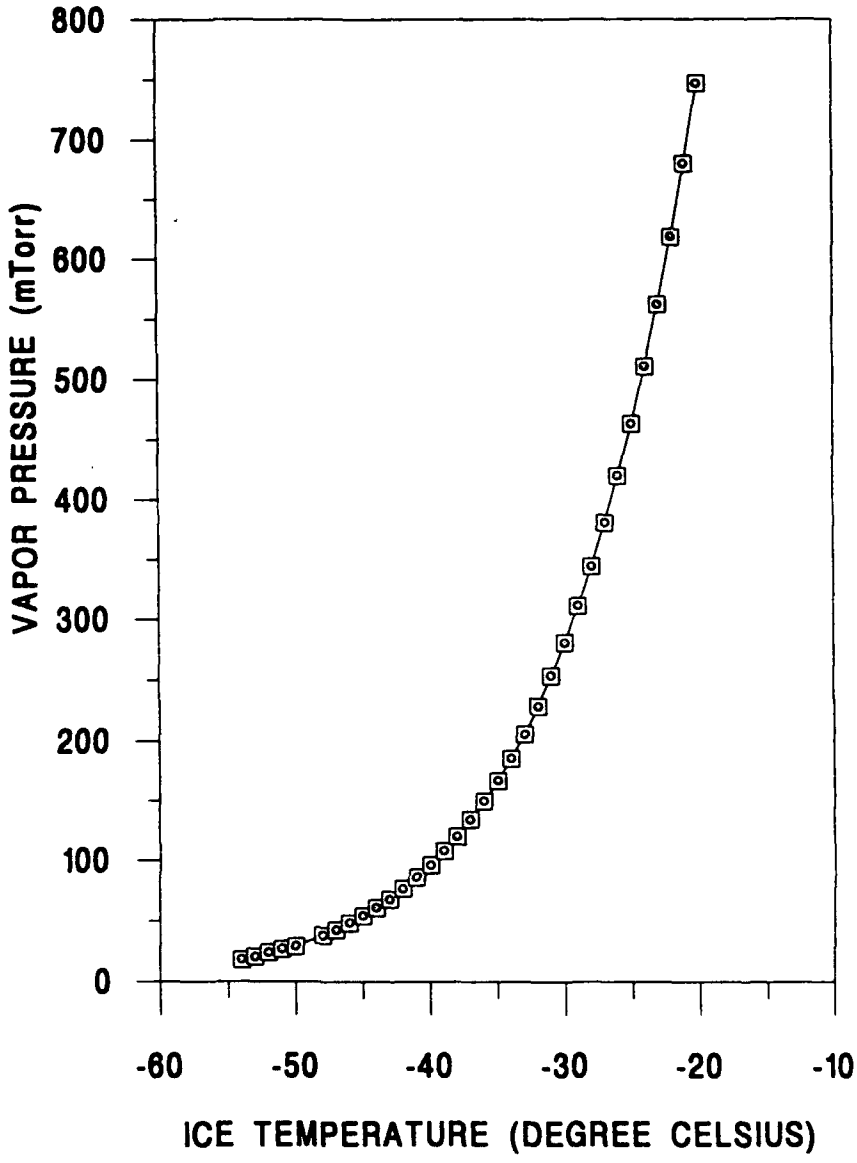


Figure 17. Vapor pressure of water as a function of ice temperature. Increasing the temperature leads to an increase in the vapor pressure of the water vapor above the ice. At temperatures below −40°C, the vapor pressure of water above the ice is very low. This leads to relatively slow drying rates since the pressure differential between the water vapor above the sample and the water vapor pressure in the condenser of the lyophilizer acts as a driving force for drying. Increases in the temperature lead to increased vapor pressures. This in turn leads to increased rates of sublimation.

observed followed the predicted glass-transition behavior of these samples very closely. A more detailed examination of the cells recovered from this process when stored for several hours at temperatures commonly used during drying was remarkable. These

Table 13. Summary of the expected target values of the characteristics of red blood cells. Note that the red blood cells were frozen at −80°C and then stored at either −80 or −38°C for 15 h.

Characteristics of normal red blood cells	Expected values	At −80°C	At −38°C
Recovery at reconstitution	90% or greater	97.6	86.9
Overall cell recovery	80% or greater	92.7	70.0
Osmotic stability of cells	70% or greater	74.6	45.8
Overall quality of cells (E)	56.0 or greater	69.2	32.1
Mean cell volume (fl)	80-100	97.0	102.89
Mean cell hemoglobin (pg)	25-35	31.8	31.2
Mean cell hemoglobin concentration (g/dl)	31-37	32.8	30.4
Maximum deformability index	0.500 or greater	0.514	0.427
Maximum cell deformability % of control	80% or greater	80.7	67.0
Osmotic deformability profiles	normal	normal	normal
Density of cell (g/ml)	1.095	1.101	1.101

cells exhibited the same loss of membrane integrity, reduction in osmotic fragility, and loss of membrane deformability as observed in the lyophilized preparations although no drying had been performed (Table 13)! Equally remarkable were the results when samples were dried in pure carbohydrate or pure polymer preparations. Although the glass-transition temperatures of these preparations were never exceeded, the results were comparable to what has been reported earlier in this text. Clearly, glass-transition theory was providing a framework for the formulation and drying protocol, but it was not indicating that the role of a water-replacement agent was not essential in the formulation.

Using the approaches of glass-transition theory and combining it with the principles of water-replacement theory, it was possible to formulate solutions that were capable of providing stability in the temperature regions of interest. These preparations normally make use of agents capable of hydrogen bonding to proteins and membrane phospholipids in the absence of water. All of our work in this area to date has been consistent with this requirement. Agents that suffice in this role include carbohydrates and polyols. In accord with the principles of glass-transition theory, these components are normally balanced with polymers that possess high, glass-transition temperatures and therefore offset the low glass-transition temperatures

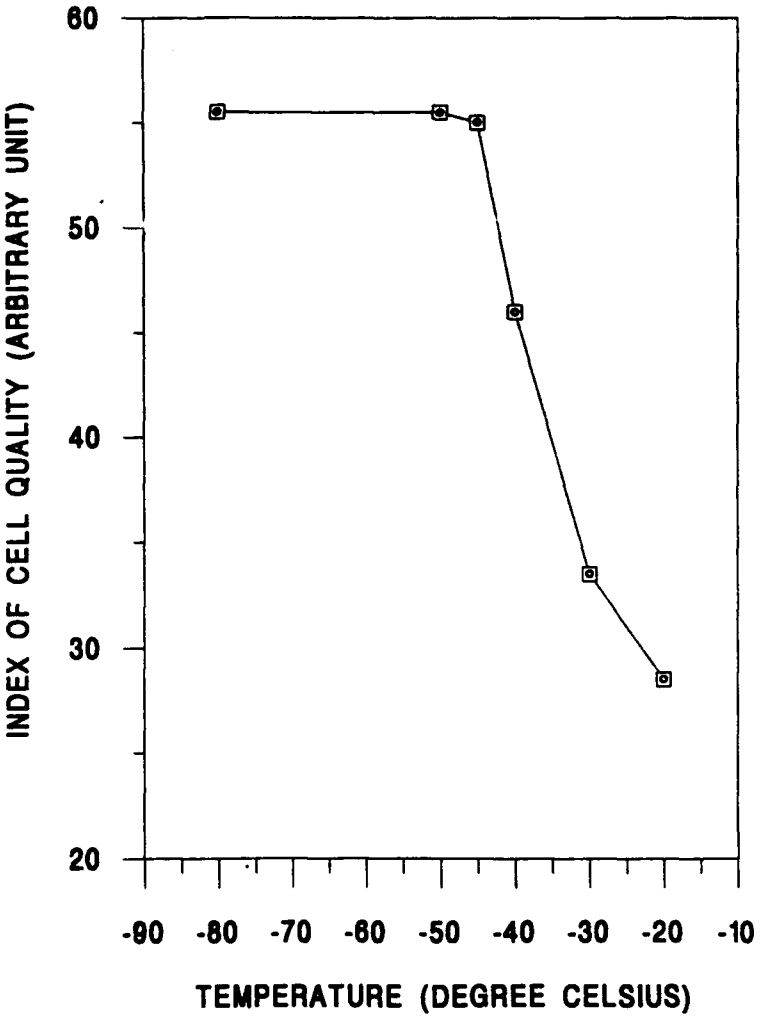


Figure 18. Quality of cells as a function of temperature of storage. The quality value was determined by the recovery of intact cells multiplied by the recovery obtained after diluting the cells 1:100 in hypotonic saline (150 mOsm) following thawing. The maximum possible value for this parameter is 100. All samples were stored at the temperatures indicated for 15–18 h. The cryoprotectant solution possessed a glass-transition temperature of −38°C. The graph clearly shows that the recovery of intact and osmotically responsive cells decreased significantly as the temperature of storage increased above this temperature. This lower recovery was also accompanied by decreases in filterability and deformability of the cells following storage.

of the low molecular weight carbohydrates and polyols. The selection of solutions with glass-transition temperatures in the range of −20 to −25°C also permits the design of drying profiles that are rapid and efficient. It is possible, for instance, to dry full units of red cells in

Table 14. Deformability characteristics of lyophilized red blood cells.

	Monkey		Human	
	fresh	lyophilized	fresh	lyophilized
Deformability index	0.56–0.60	0.56 ± 0.01	0.64–0.68	0.64 ± 0.02
Relative filtration index[a]	ND	ND	0.90–1.0	0.86±0.08

[a]Filtration rate measured through a 5 μm filter.

lyophilizer cycles requiring as little as 16-18 h for completion.

In our experience, it is possible to prepare samples with moisture contents on the order of 20–30%, as measured by the Karl–Fischer method. These preparations are stable for extended periods of storage at temperatures of 4°C and even room temperature. Our current work is focusing on defining a limit to the shelf-life of these samples under various conditions of moisture content and temperature. The level of moisture remaining in the samples appears to be high, but we have found, in accord with the principles of glass-transition theory, that it is only necessary to remove enough water from the samples to provide stability in the temperature region of interest. The remaining water in the glass is not available in a form that is capable of acting as a solvent or plasticizer for the chemical processes in the matrix as long as the glass-transition temperature is not exceeded. It is interesting to also note that the amount of water-removal tolerated under these conditions is very close to that observed for naturally occurring organisms that are also capable of withstanding conditions of dehydration.

The properties of cells produced using these methods have been reported by Sowemimo-Coker et al. (1993). Unlike previous lyophilized red cell preparations, these samples exhibit normal rheological and filtration properties (Figure 19 and Table 14). Lyophilized red cells have also been used in clinical studies involving primates. As Table 15 indicates, the data obtained from these radiolabelled red cell studies were consistent with high levels of *in vivo* survival and recovery of circulating and viable cells.

Our hope in this endeavor is to develop this technology for the long-term preservation of red cells for use in transfusion medicine in civilian and military trauma care fields. If we are successful in doing so, it will serve as a tribute to our numerous staff members,

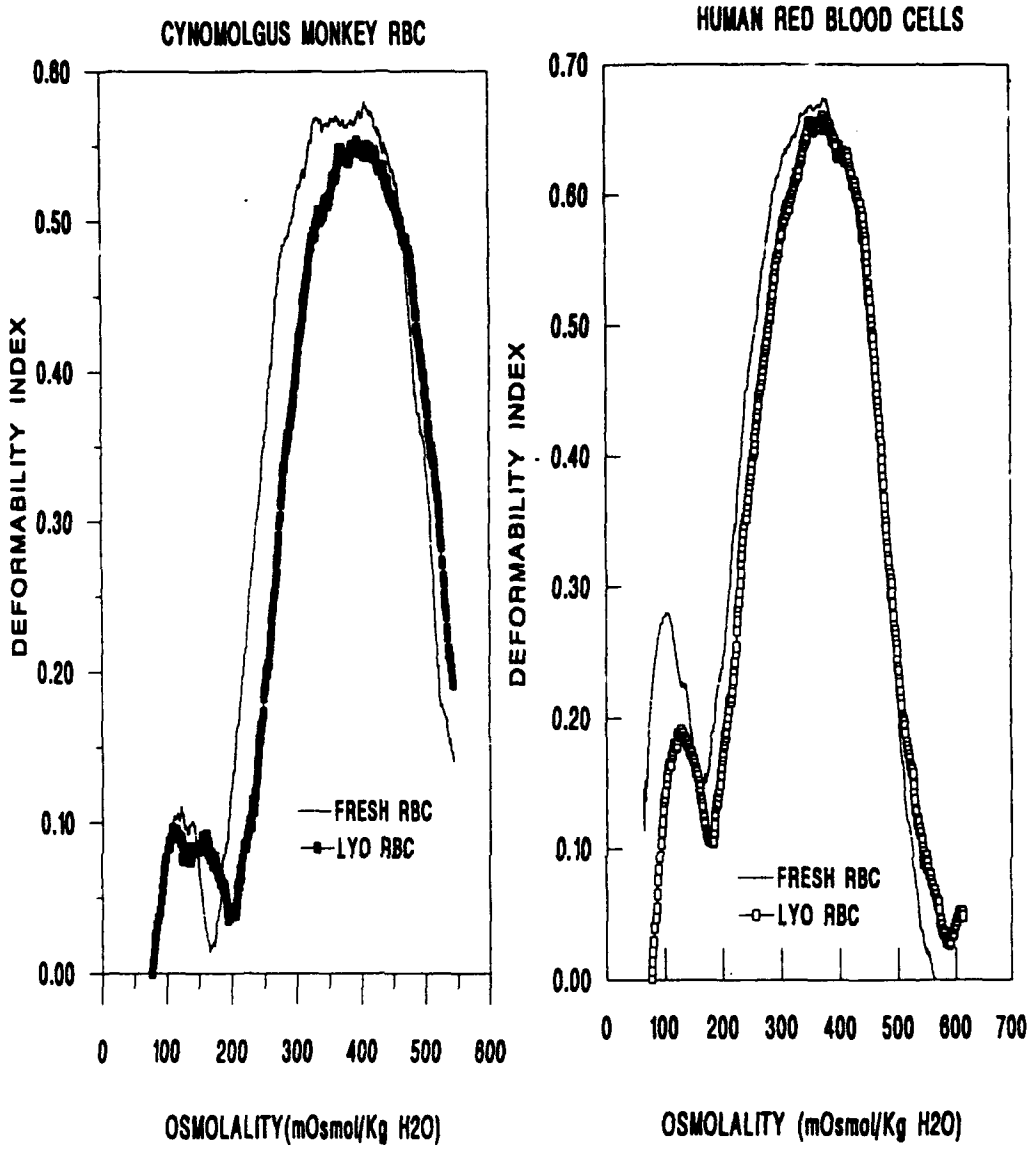


Figure 19. Deformability profiles obtained using the ektacytometer. Samples of primate and human erythrocytes were examined using an ektacytometer. The samples were subjected to a constant shear stress at different osmolalities. The resulting deformation profiles obtained by laser diffraction of the sheared cells was examined to obtain the deformability index. For both human and primate cells, the fresh and lyophilized samples exhibited comparable behavior, an indication that lyophilization did not affect the ability of the cells to deform under shear stress. Maximum deformation was observed for human cells at 300–350 mOsm and for primate cells at 350–400 mOsm indicating slight differences for the two species in surface to volume ratios. The primate samples studied in this case were from *Cynomolgus fascicularis.*

Table 15. Red cell survival studies in *Cynomolgus macaques.*

Time	LO316	B334
5 min	99.9	97.4
7.5 min	96.6	97.7
10 min	91.8	92.2
15 min	95.5	93.0
1 day	87.0	93.5
2 days	77.7	58.4
3 days	68.3	69.7
4 days	65.0	67.7
5 days	64.1	60.0
6 days	54.3	58.1
24-hour survival	87.0%	93.5%
T_{50} (in days)	8.1	10.4

Volume infused: 4–6 ml = 40–60 ml equivalent in human subject.
Values for fresh cells: 24-hour survival = 89–93%, T_{50} (in days) = 9–10 days.

collaborators, and other academicians who embarked on this task despite the odds and the skepticism of their peers (not to mention their own skepticism). It is because of people like these and their inherent scientific curiosity that what can supposedly never be done is often done.

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